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MSS. for the successive numbers of Vol. 26 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICE.

ROBERT STENHOUSE WILLIAMS (1871–1932).

THE very sudden death of Professor Stenhouse Williams on February 2nd came as a grievous shock, not only to his friends in the Biochemical Society, but also to a very wide circle in the medical, bacteriological, agricultural and dairy communities, among which his work and strong personality will long be remembered.

Stenhouse Williams started his career as a medical man. He was a Bachelor of Medicine, and Master of Surgery of Edinburgh University, and also obtained the D.P.H. of the University of Cambridge. For many years he was the Assistant Bacteriologist in the Public Health Department of Liverpool University. He also held several lectureships on public health and bacteriology in Liverpool. As Stipendiary Medical Officer at the Chest Hospital he had opportunities for studying the clinical symptoms and treatment of disease.

He had a great enthusiasm for research work and for bacteriology, and inspired groups of workers in Liverpool and afterwards in Reading with his own ardour. Although the scientific atmosphere of the Pasteur Institute, where he worked for a year, was the one most congenial to him, he still brought the same enthusiasm to bear on his co-operation with farmers and dairymen in his efforts to improve the nation's milk supply.

His research work and medical experiences in Liverpool led him to undertake studies on the sources of infection of milk by tubercle and to investigate the means which might be taken for the prevention of the spread of the disease.

He also conducted a thorough examination into the sources of contamination of milk during its passage from the cow to the consumer. It was this work and his personal influence with the dairy farmer which helped to bring about the startling improvements in the practical methods of milk production and assisted in the establishment of the Graded T.T. milks with which his name will remain associated.

The advancement of biochemical knowledge since 1912 and the recognition of the complexity of the dietary requirements of the animal body have justified the wisdom of his selection of fresh natural milk as the ideal for which the dairy industry should strive. By his bacteriological researches he has helped to make possible the practical realisation of this ideal and by so doing has enriched and ennobled the aspirations of the dairy farmer.

Another lasting memorial to his memory is the National Institute for Research in Dairying at Reading, to the development and endowment of which he devoted the last twelve years of his life. From the time he came to Reading it was obvious that a farm and adequate laboratories were necessary for the proper development of dairy research but his was an heroic effort which built up in so short a time an institute for research which already finds laboratory accommodation for over a score of workers and has gained a world-wide reputation. It was only in the last two years of his life, under pressure of growing administrative duties, that he accepted the title of Director of the Institute. To those who opposed his principles he was an unyielding antagonist, but his singleness of purpose, his justice, and loyalty to his friends, no less than his sympathy and sense of humour, cemented many friendships in the wide and varied circle of those with whom his work brought him in contact. J.G.

LXIV. STRUCTURE AND ENZYME REACTIONS.
XI. ACTION OF SALTS IN THE SYSTEMS: STARCH—AMYLASE—PROTEINS.

By GEORGE BAUMGARTEN.

From the Biochemical Laboratory, Medical Faculty, Warsaw University.

(*Received January 25th, 1932.*)

In Part X of this series [Chrempińska, 1931] the effect of the addition of electrolytes to systems containing protein, starch and amylase of vegetable origin was studied. The action of the enzyme was examined by determining the amount of residual starch, whilst in the present paper the same problem was studied, determining reducing sugars produced in the presence of taka-diastase and of ptyalin.

Experimental.

Materials used.

Enzyme. Merck's "Diastase absolut" (0·03–0·1 % solutions in 1 % NaCl.). Ptyalin: 10 cc. of filtered saliva diluted to 50 cc. with water or 1 % NaCl.

Substrate. Merck's sol. starch, solutions of which were prepared as in Part X.

Protein. Fresh or heat-coagulated egg-white was taken.

Methods.

The p_H of solutions was adjusted by addition of 0·1 *N* HCl or NaOH. The reaction temperature was 25°. Solutions were freed from protein by adding 10 cc. of Hagedorn-Jensen reagent (40 cc. 4·5 % $ZnSO_4$, 80 cc. *N* NaOH, and 120 cc. H_2O) to 20 cc. of the solution under examination. Starch is unaffected by this reagent, and maltose can be determined in the filtrate by Bertrand's method with fair accuracy (this was tested by heating 20 cc. of 0·2 % maltose (40 mg.) for 6 minutes with 10 cc. of reagent, and determining maltose in the filtrate; 39·1 and 39·4 mg. were found). In view of the fact that reducing substances pass from egg-white or ovalbumin into the solution during the reaction, a control system containing the same constituents as the experimental systems, but with water in place of amylase, was taken in all cases, and its apparent maltose content was deducted from the values obtained for the experimental systems. All results are the mean of 4–6 determinations.

Results.

A. *Taka-diastase.*

(1) p_H *8·85.* Mixtures of 5 cc. of egg-white, 10 cc. of 1 % starch, and 4 cc. of water or of solutions of NaCl, Na_2SO_4 or $CaCl_2$ were thoroughly shaken, and kept 15 minutes at 25°, after which 1 cc. of water or of 0·1 % amylase in 1 % NaCl solution was added, and the reducing sugar content was determined after 4·3 hours at 25°. The results (Table I) indicate that reaction is accelerated by

Table I. *Influence of $CaCl_2$ or NaCl on the action of taka-diastase.*

	Without NaCl or KCl							
	p_H 8·85 time 4·3 hr.		p_H 8·1 time 3·75 hr.		p_H 6·5 time 1·5 hr.		p_H 4·7 time 1 hr.	
Salt taken	mg. maltose	% acceleration	mg. maltose	% acceleration	mg. maltose	% acceleration	mg. maltose	% acceleration
—	4·33	—	32·6	—	31·25	—	33·3	—
0·005 *M* $CaCl_2$	7·77	79·4	33·3	2·1	30·35	—	32·1	−3·6
0·01 *M* $CaCl_2$	19·37	347·3	35·6	9·1	31·0	—	29·7	−10·8
0·02 *M* $CaCl_2$	25·18	481·3	38·3	17·5	31·45	—	27·25	−18·2
0·05 *M* $CaCl_2$	32·94	660·7	39·25	20·4	31·15	—	25·8	−22·5
—	4·15	—	32·6	—	31·25	—	33·3	—
0·01 *M* NaCl	5·67	36·6	33·58	3·0	32·72	4·7	34·10	+2·4
0·02 *M* NaCl	8·53	105·6	36·0	10·3	34·0	8·7	35·14	+5·5
0·05 *M* NaCl	12·28	196·0	36·76	12·8	34·9	11·6	35·6	+6·9
0·1 *M* NaCl	16·05	287·0	37·2	14·1	35·1	12·3	36·25	+8·9

	With NaCl				With KCl			
	p_H 8·85 time 6·75 hr.		p_H 8·1 time 2·5 hr.		p_H 8·85 time 7 hr.		p_H 8·1 time 2·5 hr.	
	mg. maltose	% acceleration	mg. maltose	% retardation	mg. maltose	% acceleration	mg. maltose	% retardation
—	8·1	—	31·35	—	7·5	—	31·7	—
0·005 *M* $CaCl_2$	11·48	41·7	30·0	4·2	10·5	40·0	30·85	2·7
0·01 *M* $CaCl_2$	17·54	116·5	29·8	5·0	17·15	129·0	29·45	7·1
0·02 *M* $CaCl_2$	31·06	283·5	28·65	8·6	30·15	302·0	27·6	12·9
0·05 *M* $CaCl_2$	35·23	335·0	27·47	12·4	32·85	338·0	25·77	18·7

from 79·4 % with 0·005 *M* $CaCl_2$ to 660·7 % with 0·05 *M* $CaCl_2$. The p_H of the systems containing 0·05 *M* $CaCl_2$ was reduced to 8·1. Na_2SO_4 has no influence on the action of Merck's amylase at all p_H values studied (4·7, 6·5, 8·1 and 8·85), and at concentrations of 0–0·5 *M*. The activating effect of NaCl is less marked than is that of $CaCl_2$, *viz.* from 36·6 to 287 % acceleration with 0·01 to 0·1 *M* NaCl, the p_H being reduced in 0·1 *M* NaCl to 8·8.

(2) p_H *8·1.* Similar experiments were performed on systems at p_H 8·1, reducing the time of reaction to 3·75 hours. The action of $CaCl_2$ is much less at this p_H, acceleration varying from 2·1 % with 0·005 *M* $CaCl_2$ to 20·4 % with 0·05 *M* $CaCl_2$; the p_H of the systems was reduced to 7·85 by 0·05 *M* $CaCl_2$. The action of NaCl is similarly smaller, varying from 3 to 14 % over the same range of concentrations.

(3) p_H *6·5.* At this p_H $CaCl_2$ has no appreciable action. With NaCl, an acceleration of 4·7 to 12·3 % is obtained over the range 0–0·1 *M* NaCl.

(4) p_H *4·7.* The time of reaction was in this case 1 hour. Reaction is retarded by 3·6 % with 0·005 *M* $CaCl_2$ and by 22·5 % with 0·05 *M* $CaCl_2$; with NaCl an acceleration of 2·4 to 8·9 % was observed.

B. *Action of $CaCl_2$ in the presence of NaCl or KCl.*

(1) p_H *8·85.* Na_2SO_4 has no influence on the velocity of reaction in the presence of 0·9 % NaCl or KCl. The acceleration due to $CaCl_2$ is much smaller in the presence of these salts than in their absence (Table I), varying from 41·7 to 335 % in the case of NaCl, and from 40·0 to 338 % in that of KCl.

(2) p_H *8·1.* $CaCl_2$ exerts an inhibitory action at this p_H, which varies from 4·2 to 12·4 % in the presence of NaCl, and from 2·7 to 18·7 % in that of KCl.

C. *Salivary amylase.*

A number of systems at p_H 8·1 were set up, containing 5 cc. of egg-white, 10 cc. of 1 % starch, 4·5 cc. of water, $CaCl_2$ or NaCl solution and 0·5 cc. of a 20 % solution of filtered saliva or water, and maltose was determined after 1 hour at 22°. The results (Table II) show that the reaction is retarded by

Table II. *Influence of $CaCl_2$ or NaCl on the action of salivary amylase at p_H 8·1.*

	Without NaCl or KCl		With NaCl		With KCl	
Salt	mg. maltose	% retardation	mg. maltose	% retardation	mg. maltose	% retardation
—	44·0	—	43·05	—	48·65	—
0·005 *M* $CaCl_2$	42·85	2·6	41·95	2·55	47·12	3·15
0·01 *M* $CaCl_2$	41·72	5·2	40·32	6·34	45·63	6·20
0·02 *M* $CaCl_2$	39·94	11·5	39·07	9·25	43·62	10·34
0·05 *M* $CaCl_2$	36·30	17·5	38·12	11·45	41·81	14·06
0·01 *M* NaCl	47·30	−7·5				
0·02 *M* NaCl	49·60	−12·7				
0·05 *M* NaCl	47·55	−8·1				
0·1 *M* NaCl	41·15	+6·5				

Time of reaction 1 hr.

$CaCl_2$, both in the presence and absence of NaCl or KCl. The percentage retardation over the range 0·005–0·05 *M* $CaCl_2$ varies from 2·6 to 17·5, with $CaCl_2$ and NaCl from 2·55 to 11·45, and with $CaCl_2$ and KCl from 3·15 to 14·06. NaCl accelerates reaction in concentrations of less than 0·05 *M* (maximal acceleration of 12·7 % with 0·02 *M* NaCl), and retards reaction in concentrations of 0·1 *M* (6·5 % retardation).

The action of $CaCl_2$ in systems containing egg-white in solution and heat-coagulated was next studied. The systems contained 5 cc. of egg-white, 10 cc. of starch, 9 cc. of water or $CaCl_2$ solution, and 1 cc. of 20 % saliva. The concentration of $CaCl_2$ was 0·05 *M* in the whole system. Maltose was determined after 30 minutes at 22°; 55 mg. were found in the absence of protein, 57·7 and 58·8 mg. respectively in the presence of soluble and of heat-coagulated egg-

white; in the presence of 0·05 *M* $CaCl_2$ the latter values fell to 49·3 and 49·5 mg. respectively, corresponding to retardations of 14·56 and 15·82 %. These results indicate that the action of $CaCl_2$ is the same irrespective of the state of dispersion of the protein.

Discussion.

Of the three salts studied, Na_2SO_4 is without action at all the p_H values and concentrations taken, whilst NaCl and $CaCl_2$ act differently according to the type of amylase taken, to the p_H, and to their concentration. In the case of taka-diastase, considerable activation was observed with $CaCl_2$ at p_H 8·85; this effect was considerably weaker in the presence of NaCl or KCl. At p_H 8·1 the action of $CaCl_2$ was also accelerative, although the percentage acceleration amounted to only 20, as compared with 660 at p_H 8·85; at p_H 6·5 the presence of $CaCl_2$ had no effect on the velocity, whilst at p_H 4·7 the reaction was retarded, as was also the case at p_H 8·1 in the presence of NaCl or KCl. NaCl accelerated reaction at all p_H values taken; at p_H 8·85 and 8·1 its action was, however, much feebler than that of $CaCl_2$. With salivary amylase its action may be accelerating or retarding according to its concentration.

A comparison of these results with those of Chrempińska shows that the augmented production of reducing sugars in the presence of $CaCl_2$ at p_H 8·85 is relatively much greater than is the augmented hydrolysis of starch under analogous conditions.

The accelerating action of $CaCl_2$ is in all probability due to reduction of the p_H of the medium where this is higher than the optimal, whilst retardation of reaction in acid media might be ascribed to the action of $CaCl_2$ on the enzyme. As was shown by Giedroyć and Przyłęcki [1931] the reduction of the p_H of alkaline solutions by $CaCl_2$ may be quite considerable (in this case from p_H 8·85 to 8·1), and, as will be shown in a succeeding paper, the production of reducing sugars is relatively considerably more increased than is that of non-reducing dextrins.

With salivary amylase the action of $CaCl_2$ is one of retardation, in the presence of either soluble or heat-coagulated egg-white.

The above observations on the whole indicate that the formation of compounds between the enzyme and substrate is not, at least in the presence of high concentrations of enzyme, of any great importance. As to the rôle of structure, this would probably only come into account in cases in which parts of the system containing enzyme are mechanically separated from others containing substrate, either by occlusion or by the interposition of difficultly permeable partitions.

This research was carried out at the suggestion and under the direction of Prof. St J. Przyłęcki, to whom the author wishes to express his gratitude for advice and assistance.

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LXV. STUDIES ON THE CATALYTIC OXIDATION OF URIC ACID.

By ZOFJA DOBROWOLSKA.

From the Biochemical Laboratory, Medical Faculty, the University, Warsaw.

(*Received February 18th, 1932.*)

Whilst numerous papers have been published on the oxidation of uric acid in the presence of metallic catalysts, such as copper, manganese, *etc.*, little work appears to have been done on the action of hydrogen acceptors on uric acid. In this paper the action of thioglycollic acid, cysteine and methylene blue will be described.

The results obtained with thioglycollic acid differ from those described in an earlier paper [Dobrowolska, 1928]; in this earlier research thioglycollic acid was found to catalyse the oxidation of uric acid. The earlier work was done with an old preparation of Kahlbaum's, which apparently contained some impurity catalysing the oxidation of uric acid, as Merck's pure thioglycollic acid in no case catalysed the reaction in question.

Experimental.

Kahlbaum's or Merck's pure or analytical reagents were taken. The solutions of thioglycollic acid were neutralised to p_H 7 with NaOH before final dilution. Uric acid solutions were prepared by dissolution in the minimal quantity of saturated lithium carbonate and adding water to the requisite dilution. Uric acid was in most cases determined colorimetrically, using Folin's reagents [Folin and Denis, 1913]. p_H was determined colorimetrically (Clark and Lubs's indicators) or potentiometrically, using a hydrogen electrode.

Thioglycollic acid.

The action of thioglycollic acid on uric acid was studied at various p_H values in the following way. A series of systems, each containing 10 cc. of 0·05 % thioglycollic acid, 0·2–0·6 % uric acid, and buffer solution (Sørensen's phosphates below p_H 8 and Palitzsch's borates above p_H 8), was set up, together with two control series, containing 10 cc. of water in place of thioglycollic or uric acid respectively. A separate control containing only buffers and thioglycollic acid was necessitated by the circumstance that this substance gives a coloration with Folin's reagents. Toluene or thymol crystals were added in order to ensure sterility of the systems. The intensity of coloration given by

every system was determined at the beginning of the experiment, and after various periods of time (the reaction temperature was 37°), and the sums of the two controls were compared with the values obtained for the experimental systems. The intensities of coloration are expressed as mg. of uric acid in Table I, from which it appears that at no p_H from 6·1 to 9·8 does any oxidation of uric acid take place. At the same time it is evident that the intensity of coloration of the systems is greater immediately after adding thioglycollic acid to uric acid than would correspond to the sum of that given by each constituent separately. Thus, at p_H 7·8, the intensity of coloration of the system corresponds to 39·2 mg. of uric acid, that of uric acid alone to 12·6, and that of thioglycollic acid to 6·0, giving a total of 18·6 mg., which is less than half of the value found for the whole system. The coloration given by thioglycollic acid alone rapidly diminishes in intensity with time, as a result of oxidation by atmospheric oxygen to dithioglycollic acid; at the same time the coloration given by the whole system approaches that given by uric acid alone, the velocity of this process being greater the higher is the p_H of the system.

Table I. *Action of thioglycollic acid on uric acid.*

			Control systems				
No. of exp.	Time hrs.	p_H	Uric acid found mg. *a.*	Thio-glycollic acid* *b.*	Sum of $a+b$ mg.	Apparent uric acid content mg.	Apparent change in uric acid content mg.
1	0	7·8	12·6	6·0	18·6	39·2	20·6
	24	7·8	12·6	4·4	17·0	20·0	3·0
	48	7·8	12·6	3·2	15·8	16·7	0·9
	72	7·8	12·6	0	12·6	14·2	1·6
	84	7·8	12·6	0	12·6	14·1	1·5
2	0	9·2	12·6	0·4	13·00	20·0	7·00
	48	9·2	12·6	0·28	12·88	14·6	1·72
	72	9·2	12·6	0·16	12·76	12·6	0·16
	96	9·2	12·2	0·16	12·36	12·6	0·24
3	0	9·8	17·8	0·10	18·20	30·8	12·6
	24	9·8	17·8	0·28	18·08	20·0	1·92
	48	9·8	16·6	0·28	16·88	16·8	0·08
	72	9·8	15·4	0	15·40	16·0	0·6
4	0	8·0	6·4	2·4	8·80	14·6	5·8
	24	8·0	6·4	2·4	8·80	11·4	2·6
	48	8·0	6·4	0	6·40	6·0	0·4
	72	8·0	6·4	0	6·40	6·0	0·4
5	0	6·1	8·0	1·8	9·80	14·6	4·8
	24	6·1	8·0	0·8	8·8	8·0	0·8
	48	6·1	8·0	0	8·0	8·0	0

* Expressed as the number of mg. of uric acid which would give an equal intensity of coloration with the Folin-Denis reagent.

No explanation of the above phenomenon is evident; possibly an unstable double compound is formed between the two acids, of a higher reduction potential.

In systems the p_H of which was below 6 or above 10, uric acid crystallised out as free acid and as sodium salt monohydrate respectively—for this reason the action of thioglycollic acid was studied only within these p_H limits.

Action of thioglycollic acid in the presence of Fe and Cu salts.

Warburg and Sakuma [1923] found that the oxidation of sugars in the presence of cysteine depended on the presence of traces of iron or copper. That this is not the case with thioglycollic acid was shown by experiments in which iron (0·0001–0·0007 *M* ferrous ammonium sulphate) or copper (0·00005–0·0001 *M* $CuSO_4, 5H_2O$) was added to systems similar to those described above. The only effect observed was an intensification of the coloration given by thioglycollic acid with the Folin-Denis reagent at the beginning of the reaction with subsequent rapid disappearance of the coloration with conversion into dithioglycollic acid by atmospheric oxygen.

Verification of results obtained by the colorimetric method.

In view of the relative unreliability of the results obtained by the colorimetric method in such systems as the above, and of the possibility that the product of action of thioglycollic acid on uric acid also gives a coloration with the Folin-Denis reagent, it was thought desirable to verify them using the following methods.

(1) *Determination of CO_2 by Van Slyke and Cullen's method.* Should uric acid undergo decomposition to allantoin, the CO_2 content of the experimental systems should be greater than that of the component solutions taken separately. No difference was observed in systems of p_H 8·0–9·8, which were isolated from atmospheric CO_2.

(2) *Determination of NH_3 and of urea.* These were determined in the experimental and control systems by Folin's and by Marshall's methods. Only insignificant traces were found in all systems, indicating that decomposition of uric acid with production of NH_3 or urea does not take place under the given conditions.

(3) *Determination of uric acid by Krüger and Schittenhelm's method, and by iodimetric titration.* The results given by these methods similarly indicate that no oxidation of uric acid takes place in the presence of thioglycollic acid.

Cysteine.

Cysteine, prepared by reduction of Kahlbaum's cystine, was without action on uric acid, either alone or in the presence of Cu, at p_H 8·0–9·1.

Methylene blue.

Grubler's methylene blue (this will be designated in future as M.B.) was taken, in concentrations of 0·000077–0·0001 *M*. Such low concentrations were taken for two reasons, firstly, that the coloration due to the dye should not interfere with the colorimetric determination of uric acid, and secondly that at higher concentrations M.B. is precipitated at p_H 10·5–12·0 in the form of long violet-blue needles.

(1) *Action under aerobic conditions.* Preliminary experiments at p_H 8 indicated that in the presence of 0·00031 *M* M.B. over 12 mg. of uric acid are decomposed at 18° within 48 hours in the absence of antiseptics. Toluene was found to exert no inhibitory action at p_H values below 9·3; in more alkaline solutions M.B. is converted into a red modification, soluble in toluene, which extracts it from aqueous solution, and thus prevents its action on uric acid. As a result the velocity of reaction varies inversely with p_H and with the volume of toluene added. When only a small volume of toluene was added (2 drops) the results obtained were substantially the same as in its absence. Thymol acts similarly, except that velocity of reaction is greater at p_H 11·6 than at p_H 9·3; in this case M.B. accumulates on the surface of the crystals of thymol and then slowly diffuses into the interior. These results indicate that toluene in small quantity does not interfere with the reaction.

A number of systems was set up, containing 10 cc. of uric acid solution, 10 cc. of 0·00031 *M* M.B. or water and 10 cc. of Sørensen's or McIlvaine's buffers, 10 cc. of 0·008 or 0·016 *M* KCN or water and 2 drops of toluene. The potassium cyanide solution was previously brought to p_H 7 by addition of *N* HCl. Uric acid was determined colorimetrically after 14–24 hours at 18°. The results, given in Table II, show that, on the whole, oxidation is greater the higher the p_H of the system, and that cyanide has practically no effect at any p_H.

Table II. *Influence of* p_H *and KCN on oxidation of uric acid in presence of methylene blue.*

			Mg. uric acid				
				Found		Decomposed	
Time hrs.	p_H	Concentration of KCN	In control	Without KCN	With KCN	Without KCN	With KCN
24	6·11	*M*/500	18·8	18·8	18·8	0	0
	6·79		18·8	18·8	18·8	0	0
	7·47		18·8	14·6	15·8	4·2	3·0
	8·35		18·8	10·0	10·6	8·8	8·2
	9·37		18·8	11·4	11·4	7·4	7·4
	9·75		18·8	9·4	9·4	7·8	7·8
	11·20		17·2	9·4	9·4	7·8	7·8
	11·75		16·4	5·2	5·2	11·2	11·2
15	6·30	*M*/500	10·8	9·6	10·4	1·2	0·4
	7·02		10·8	8·8	8·8	2·0	2·0
	7·70		10·8	5·3	6·9	5·5	4·9
	8·60		10·8	4·4	4·9	6·4	5·9
	9·75		10·8	4·9	5·5	5·9	5·3
	10·15		10·8	4·6	5·0	6·2	5·8
	11·85		8·6	2·94	2·54	5·66	6·06
	12·30		8·6	1·32	1·16	7·28	7·44
14	5·75	*M*/250	13·2	10·4	10·4	2·8	2·8
	6·40		13·2	8·01	9·2	5·1	4·0
	7·00		13·2	6·7	7·1	6·5	6·1
	7·82		13·2	5·6	6·0	7·6	7·2
	8·84		13·2	4·04	5·6	9·16	7·6
	9·27		13·2	4·6	5·5	8·6	7·7
	10·75		12·4	3·14	3·14	9·26	9·26
	11·25		12·4	1·82	1·82	10·58	10·78

Oxidation augments sharply from p_H 6 to about 9·6, above which it falls to p_H 10, again rising steadily to p_H 12. In the presence of cyanide a break occurs in the oxidation-p_H curve at about p_H 8·6, above which value the number of mg. of uric acid oxidised increases at first slowly and then rapidly to attain at p_H 11–12 the same value as in the absence of cyanide.

The action of M.B. in the presence of Cu and Fe ions was next studied. Systems similar to the above were prepared, with the difference that copper sulphate or ferrous ammonium sulphate solution was added in place of KCN.

It was found that 0·0001 *M* ferrous ammonium sulphate did not influence the velocity of reaction between p_H 5·7 and 12·0.

Copper alone catalyses the oxidation of uric acid, in the absence of M.B.; where both are present together they exert a mutually antagonistic action, which is more marked at higher than at lower p_H. Thus, at p_H 12·1, 7·5 mg. are oxidised in the presence of copper alone, and 6·74 mg. in the presence of M.B. alone; in the presence of both M.B. and Cu only 6·34 mg. of uric acid are oxidised (Table III). The influence of Cu on the action of M.B. is probably due to the formation of a less active compound of the two substances, and, at certain p_H values (7–10), a deposit of dark blue needles was observed in the systems, with partial decoloration of the solution.

Table III. *Influence of 0·0001* M *copper sulphate on oxidation of uric acid by methylene blue at 21°.*

		Mg. uric acid						
		In absence of $CuSO_4$			In presence of $CuSO_4$			
Time hrs.	p_H	In control	In system	De-composed	In control	In system	De-composed	Inhibition mg.
14½	5·87	11·2	11·2	0	11·2	11·2	0	0
	6·79	11·2	10·8	0·4	11·2	11·2	0	0·4
	7·47	11·2	8·1	3·1	10·2	10·2	0	3·1
	8·37	11·2	5·0	6·2	10·0	6·5	3·5	2·7
	9·55	11·2	6·3	4·9	10·0	7·5	2·5	2·4
	10·1	11·2	6·4	4·8	8·8	7·3	1·5	3·3
	11·8	9·0	4·05	4·95	3·65	4·6	0	4·95
	12·1	9·8	3·06	6·74	2·3	3·46	0	6·74
14	5·81	12·2	12·2	0	12·2	12·2	0	0
	6·65	12·2	11·6	0·6	12·0	12·0	0	0·6
	7·34	12·2	9·2	3·0	11·6	11·8	0	3·0
	8·14	11·8	7·4	4·4	11·8	8·8	3·0	1·4
	9·3	11·8	6·2	5·6	10·0	7·3	2·7	2·9
	9·8	10·6	4·8	5·8	9·2	6·3	2·9	2·9
	11·45	8·8	3·65	5·15	2·75	4·4	0	5·15
	12·0	9·6	2·3	7·3	2·88	3·35	0	7·3

It was observed that freshly prepared solutions of M.B. exerted a considerably smaller catalytic action than did aged solutions. Thus in one case, in a system containing 10 cc. of uric acid solution (18 mg.), 10 cc. of freshly dissolved M.B., and 20 cc. of buffers (p_H 8·2), 0·14 mg. of uric acid was decomposed during 24 hours at 23°, whilst in a similar system containing a 20-day old solution of M.B. 8·4 mg. were oxidised in 24 hours; after 20 days the activity of M.B.

solutions does not appear further to increase. This activation can be accelerated by alkalising the M.B. solution, as is shown by the following experiment. Part of a solution of M.B. was alkalised with NaOH to p_H 11·6, and left 3 days, when the p_H was reduced to 4·74 by addition of HCl. Two sets of systems were then set up, previously alkalised M.B. being taken in one set, and ordinary M.B. in the other. The results, given in Table IV, indicate that an activation of about 20 % is attained in the above way.

Table IV. *Activation of methylene blue.*

		Mg. uric acid					
Time hrs.	p_H	In control	With ordinary methylene blue	De-composed	With methylene blue	De-composed	% activation
22½	8·5	11·0	9·2	1·8	6·9	4·1	21
22	8·7	11·8	8·6	3·2	3·4	5·46	20

(2) *Action of M.B. under anaerobic conditions.* Systems containing uric acid, M.B. and buffers (p_H 6–11·4) were left in Schroetter fermentation vessels for 43–69 hours at 23°, atmospheric oxygen being excluded by sealing the side tube with mercury.

Under these conditions no oxidation of uric acid took place. That reaction was inhibited by exclusion of oxygen and not by the action of mercury on M.B. was shown by similar systems left in contact with the air in the presence of mercury; no retardation of reaction was observed in these circumstances.

Discussion.

The inactivity of sulphydryl compounds, and of methylene blue under anaerobic conditions indicates that uric acid cannot be oxidised by dehydrogenation under these conditions, but only by direct addition of oxygen. Methylene blue is probably itself inactive even under aerobic conditions—the actual catalyst is most probably some product of methylene blue, capable of combining with atmospheric oxygen, and of donating this oxygen to uric acid. Whether or not iron or copper enters into the composition of this catalyst it is not possible, on the basis of the above experimental results, to state with certainty; the fact that cyanide does not inhibit reaction is not evidence that metals are absent, as haemoglobin is capable of catalysing the oxidation of uric acid in the presence of cyanide [Gomolińska, 1928]. The absence of activation on addition of Cu or Fe salts similarly does not exclude the possibility that the presence of traces of metal may be essential to the action of M.B., as was found to be the case for cysteine [Warburg and Sakuma, 1923].

The nature of the red modification of M.B. obtained in alkaline solutions is similarly not clear. Commencing from p_H 9, an increasing proportion of a red, ether-soluble modification is found, and at p_H 12 practically the whole of

the dye is extractable from aqueous solution by organic solvents. The ether extract on shaking with distilled water loses its red coloration, and the aqueous layer becomes coloured blue, indicating that a labile equilibrium ordinarily exists between the two forms, the mutual proportions of which depend on the p_H. The increase in oxidation with p_H may be due to the increasing concentration of the red form.

The observation that the coloration given by mixtures of uric acid and sulphydryl compounds is greater than the sum of those given by each substance separately is of importance in the application of colorimetric methods to the determination of cysteine, ergothioneine, *etc.*, in the presence of uric acid, or *vice versa*. Unless Folin's isolation procedure is applied for the removal of uric acid the results obtained for most physiological fluids would be untrustworthy, and the same applies to the determination of uric acid in solutions in which processes of decay have taken place, with the production of hydrogen sulphide.

Summary.

1. The sulphydryl compounds thioglycollic acid and cysteine, and the disulphide compounds dithiodiglycollic acid and cystine are without influence on the oxidation of uric acid, either in the presence or absence of salts of iron or copper.

2. The coloration given on the addition of the Folin-Denis reagent to a system containing uric acid and a sulphydryl compound is more intense than the sum of the colorations given by each constituent separately; this extra coloration diminishes with increasing p_H. A similar effect is observed in systems containing thioglycollic acid and copper sulphate.

3. Monohydrated sodium urate separates from systems containing uric acid and thioglycollic acid at p_H 10·5–11·5.

4. Methylene blue catalyses the reaction of oxidation of uric acid in aerobic but not in anaerobic conditions; the velocity of reaction varies with the p_H of the medium.

5. The reaction is inhibited to a varying extent, according to the p_H of the medium, by copper salts (0·0001 *M* copper sulphate).

6. The reaction is to a slight extent inhibited by 0·004 *M* potassium cyanide over the range p_H 6–10. At p_H 11 and 12 the cyanide undergoes hydrolysis, with consequent loss of inhibitory action.

7. Crystalline thymol, added as an antiseptic, retards the reaction, as a result of adsorption or solid solution of methylene blue.

8. The addition of toluene as an antiseptic similarly retards the reaction, as a result of the solubility in organic solvents of a red variety of methylene blue, formed in alkaline solution, p_H 10–12.

9. The catalytic activity of methylene blue rises considerably during the first few weeks after preparation of its dilute solutions; this process is completed more rapidly in alkaline than in other solutions.

10. The reaction of oxidation of uric acid in the presence of methylene blue appears to be one of catalytic oxidation, oxygen being in all probability activated by methylene blue.

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LXVI. IS THERE A CO-ENZYME FOR UREASE?

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When it was discovered in this laboratory [Sumner, 1926, 1, 2] that jack bean urease could be separated as globulin crystals and that these could be recrystallised without loss of activity we naturally believed this evidence sufficient to prove that no co-enzyme for urease could exist. For it was to be expected that crystallisation would separate the urease from its co-enzyme, if such were present in the jack bean, and that crystalline urease would be partly inactive and would become still less active on recrystallising. On the contrary, recrystallisation from both 32 % acetone and from 30 % alcohol has been found to cause an increase in activity instead of a decrease. Moreover, it was noted at the outset that the decrease of enzyme activity that occurs when the crystals separate from a 32 % acetone jack bean extract is almost exactly equal to the activity of the crystals themselves, and a statement was made at that time that no co-enzyme for urease could be present in the jack bean [Sumner, 1926, 1]. Nevertheless, co-urease has been discussed in a recent monograph on enzymes without any mention being made of the practical certainty that there is no such substance [Haldane, 1930]. We have therefore considered it necessary to repeat some of the experiments of Onodera [1915] and Kato [1923], who are responsible for the claim for the existence of co-urease. It might be argued that our findings apply only to jack bean urease and that they are not valid for soya bean urease, which was the enzyme used by the authors mentioned above. However, we have shown recently that soya urease is inhibited by highly purified jack bean anti-urease and that it gives the precipitin test with this anti-urease also, which establishes an immunological relationship between the two enzymes [Kirk and Sumner, 1932].

Onodera [1915] brought forward evidence for the existence of a co-enzyme for soya urease, although he states: "Indeed its nature, as will be seen, throws some doubt on its claim to the title." He prepared soya urease according to the method of Van Slyke and Cullen [1914]. Urease dialysed for 5 days against distilled water lost nearly all its activity, but on adding to this some fresh urease the lost activity was partly restored. No co-urease could be found in the dialysate. Urease treated with acid, or alkali, or heated even to boiling lost its co-enzyme, while the urease proper was not destroyed, as could be deduced from the fact that addition of fresh urease partly restored the activity.

As the result of experiments which need not be discussed here Onodera further claimed that the co-enzyme consisted of two substances, one dialysable and the other not dialysable.

Kato [1923] stated that soya urease preparations contain two essential constituents, the enzyme proper and an unknown substance *X*. He believed substance *X* to react with urea to form a compound which could be hydrolysed by urease. He stated that urea could not be hydrolysed in the absence of substance *X* and that urease proper was inactivated by urea if *X* were absent. Kato used phosphate buffer in certain experiments for determining urease activity and in other experiments he used no buffer. He claimed that it was impossible to compare the activities of two different urease preparations and showed (his Table I) how various quantities of preparation A and of preparation B gave values for urease content that fluctuated in a most extraordinary manner. Here no buffer was employed.

By treating soya urease with freshly precipitated calcium phosphate he obtained two fractions, a filtrate and a precipitate. The filtrate alone had a weak urease activity and this was not increased by adding glycine. The precipitate had by itself a weak urease action and this was increased by adding either some glycine or some of the filtrate. In determining the urease action he used no buffer (his Table 36).

Lövgren [1921] has repeated some of Onodera's experiments, using phosphate buffer, and has concluded that Onodera's claim for co-urease is unconvincing and that the results are to be explained as due principally to a p_H effect.

Our first explanation of the results of Onodera and Kato was the same as Lövgren's. For one could expect that urease *plus* inactivated urease would decompose urea more rapidly than urease alone, since the inactivated urease would serve as buffer to some extent and would maintain a more favourable p_H. However, we have tested this out experimentally and have found that while urease *plus* inactivated urease always hydrolyses more urea than does urease alone, the difference is slight. Table I illustrates this.

Table I.

	Ammonia-N (mg.)
5 cc. Van Slyke soya urease heated 10 mins. in boiling water. Added 45 cc. redistilled water. Added 1 cc. 0·5 % urease. Brought to 30° in bath. Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl. Analysed for ammonia by aeration	16·5
To 1 cc. 0·5 % soya urease added 50 cc. redistilled water. Brought to 30° in bath. Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl	15·4

The buffering power of soya urease solution is small and the results of Onodera and Kato cannot be accounted for as due solely to p_H effect. However, there is a more plausible reason for their findings. This is the protective effect of the colloids present in inactivated urease against traces of heavy metals in distilled water. The experiments in Table II show how great a difference is

made when a minute amount of copper sulphate is added on the one hand to soya urease *plus* boiled urease and, on the other hand, to soya urease containing no boiled urease.

Table II.

	Ammonia-N (mg.)
5 cc. 5 % Van Slyke soya urease heated 10 mins. in boiling water. Added 44 cc. redistilled water. Added 1 cc. 0·5 % urease and 1 cc. containing 0·035 mg. $CuSO_4$. Brought to 30° (15 mins.). Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl	16·0
To 1 cc. 0·5 % urease added 49 cc. redistilled water and 1 cc. 0·035 mg. $CuSO_4$. Brought to 30° (15 mins.). Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl	0·13

In Table III the same procedure was followed except that 5 cc. of 9·6 % neutral phosphate were added in place of 5 cc. of the water.

Table III.

	Ammonia-N (mg.)
5 cc. 5 % Van Slyke soya urease heated 10 mins. in boiling water. Added 39 cc. redistilled water. Added 1 cc. 0·5 % urease, 1 cc. containing 0·035 mg. $CuSO_4$ and 5 cc. phosphate. Brought to 30° (15 mins.). Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl	30·1
To 1 cc. 0·5 % urease added 44 cc. redistilled water, 1 cc. containing 0·035 mg. $CuSO_4$ and 5 cc. phosphate. Brought to 30° (15 mins.). Added 10 cc. 3 % urea. After 4 hours added 25 cc. *N* HCl	0·86

It is of course impossible to be certain that the distilled water employed by Onodera and Kato contained traces of heavy metal, but it is very probable that it did. We have carried out experiments both with crystalline urease and with impure soya bean urease in which the water employed was, as usual, redistilled from glass. Our analyses, as will be seen below, show that when dialysed urease is added to fresh urease the mixture possesses exactly the sum of the activity of the two components.

All the glassware was free from adsorbed films of heavy metal. Our reagents were carefully purified to assure freedom from heavy metals. The method for estimating urease activity was the one employed in this laboratory for the past 4 years [Sumner and Hand, 1928]. Crystalline urease was prepared by our usual method. Soya urease was prepared by extracting 1 part of defatted, dust-fine meal with 5 parts of water for 1 hour, followed by centrifuging. The soya beans were first ground coarsely in a coffee mill equipped with steel burrs, then separated from the hulls by an air blast and extracted with light petroleum. After evaporating off the solvent the material was ground in a porcelain ball mill and sifted through silk bolting cloth.

Table IV.

Crystalline jack bean urease 1 cc. (1140 units) was dialysed under toluene in a collodion bag against 4 changes of water (450 cc. each) in an ice-chest for 72 hours. The material was washed with a little neutral phosphate and diluted to 50 cc. The solution was turbid, showing denaturation. 10 cc. were diluted to 92 cc. and this solution was tested for activity both alone and after mixing with an equal vol. of fresh diluted urease.

	Urease units
1 cc. dialysed diluted urease	0·93
1 cc. fresh diluted urease ...	1·07
1 cc. mixture 1:1	0·98

Calculation shows that during dialysis the urease lost 62 % of its activity. If the activity of the dialysed urease had been completely restored by addition of fresh urease the mixture should have contained 1·75 units per cc. However, it had an activity of only 0·98 unit, while the average of the two components gives 1·00.

Table V.

5·0 cc. of soya urease were dialysed under toluene in the ice-chest 15 days. The water (450 cc.) was changed every day. During this time the soya urease lost 55 % of its activity. Presumably the reason why the loss was not greater was because the water was free from heavy metals. The material was washed out with neutral phosphate and diluted to 42 cc. 5 cc. of the original urease were diluted to 42 cc. and the 2 solutions were tested for urease activity both separately and after mixing 1:1.

	Urease units	
1 cc. diluted dialysed urease	0·21,	0·23
1 cc. fresh diluted urease ...	0·49,	0·48
1 cc. mixture 1:1	0·36,	0·36

Here again the activity of the mixture, 0·36 unit, is almost exactly equal to the sum of the activities of the two components, 0·35 unit. If any co-urease were present this would not be true.

Conclusion.

Active urease *plus* inactivated urease produces only slightly more ammonia from urea than active urease alone.

If traces of heavy metal are present urease is inactivated less readily in the presence of boiled urease than in its absence.

The results of Onodera [1915] and Kato [1923] cannot be accepted as an indication of the presence of a co-urease in their preparations, but are probably to be explained as due to a protection of urease against heavy metal impurities.

Our experiments with crystalline urease and soya urease show that there is no evidence of a co-enzyme for urease.

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LXVII. CHOLEIC ACIDS.

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I. THE BIOCHEMICAL SIGNIFICANCE OF THE CHOLEIC ACID PRINCIPLE.

Physical properties in homologous series are functions of molecular size. When constants like melting-point, solubility, viscosity, surface tension effects, acid dissociation constant and others are plotted against molecular weight or against the number of carbon atoms, the resulting diagrams indicate regular gradual changes of these physical properties, *e.g.* the solubilities of the ethyl esters in the acetic acid series were found to follow an inverse geometrical progression with the length of the carbon chain [Sobotka and Kahn, 1931]. Some properties follow an oscillatory course between those homologues with an even number of carbon atoms and those with an odd one. Their physical and chemical properties will be reflected by their biochemical behaviour. Diffusion through membranes, toxicity towards cells, specific pharmacological effects, and even the probability of their formation and occurrence depend on the combined effects of simpler physical constants.

Nevertheless, not all biochemical properties of homologous substances can be explained on the basis of these additive factors. The synthesis of fatty acids from carbohydrates may be associated with preferential formation of fatty acids whose number of carbon atoms is a multiple of six. Another factor which might induce periodical, instead of gradual changes, in the biochemical properties of homologous fatty acids and their derivatives is their "co-ordinative valency," as revealed by the study of the choleic acids.

The central position of one atom or ion surrounded by a number of molecules has been recognised as the general architectural principle of inorganic molecular compounds. This number must be such as to allow for a symmetrical arrangement around the pivotal atom and the co-ordination numbers most commonly encountered are 4, 6, 8, occasionally 2 and 3, while the numbers 5 and 7 would not permit symmetrical constellation. Potential higher co-ordination numbers are 12 and 20 [Hüttig, 1920] but no evidence has been adduced for the existence of such huge symmetrical molecular aggregates.

Organic molecular compounds are of a much simpler structure, the prevailing molecular ratio being 1 : 1 in almost every instance. The choleic acids form the most remarkable exception. Wieland and Sorge [1916] recognised that natural "choleic acid" was a molecular compound consisting of 8 molecules of

desoxycholic acid and 1 molecule of stearic or palmitic acid. While all bile acids are known to influence the physical condition of lipoids in the dissolved state, desoxycholic acid seems to have a special chemical affinity for fatty acids and other substances, giving rise to the formation of crystalline molecular compounds. This principle, designated the "Choleic Acid Principle," shed new light on the significance of the bile in metabolism. Wieland and Sorge recognised that molecular compounds of desoxycholic acid of this type may be formed not only with ethyl alcohol, ether, acetone, acetic and higher fatty acids, but also with substances like benzene, phenol, naphthalene, camphor and others, rendering them water-soluble in the form of alkali choleinates, a fact of no mean physiological and pharmacological importance.

Choleic acids in homologous series. Rheinboldt and his co-workers [1926, 1929] studied the choleic acids consisting of aliphatic acids, and their esters, with desoxycholic acid or Boedecker's apocholic acid. They reached the following conclusions. (1) Choleic acids are built on the co-ordination principle and show the same molecular ratios as inorganic co-ordination compounds and as postulated by geometric considerations. They are "co-ordination compounds of higher order." (2) One molecule of acetic acid combines with a single molecule of desoxycholic acid, 1 molecule of propionic acid with 3 molecules of the bile acid, butyric to caprylic acids with 4. For pelargonic to myristic acids the co-ordination number is 6, for pentadecylic acid upwards 8. The same numbers obtain for analogous acids with double bonds. (3) An alcohol with n carbon atoms has the same co-ordination number as the acid with $(n + 1)$ carbon atoms. (4) In alkyl esters of aliphatic acids the co-ordination number peculiar to the longer of the two chains prevails.

A graphic representation of the data mentioned under (2) yields a terrace-shaped diagram (Fig. 8). In the group of choleic acids between butyric and caprylic acids one may expect a steady increase of "co-ordinative unsaturation" while a new cycle will be resumed with pelargonic acid-choleic acid. Thus a formal resemblance to the periodic system of elements is suggested.

Since the bearing of co-ordinative valency upon biochemical problems is being studied in this laboratory, it seemed desirable to secure data on the co-ordination numbers in other homologous series of biochemical significance. In Part II the choleic acids derived from saturated aliphatic dicarboxylic acids are described.

Choleic acids of isomerides. Other aspects of the Choleic Acid Principle are offered by the study of choleic acids derived from isomeric substances. The choleic acids of the isomeric valeric acids, to be dealt with below (Part III), exemplify the influence of structural isomerism. Similar considerations actuated experiments to decide whether or not the bile acids permit one to differentiate between optical antipodes. Successful experiments in this direction will be reported in a subsequent paper [Sobotka, 1931; Sobotka and Goldberg, 1932]. We are also studying the relation of *cis-trans* isomerism on choleic acid formation.

An investigation of ethyl acetoacetate-choleic acid [Sobotka and Kahn, 1932] and the choleic acids of related tautomeric substances yielded results of twofold interest. We could demonstrate that ethyl acetoacetate in its molecular compound with three molecules of desoxycholic acid is completely enolised. This finding suggests a participation of the bile acids in the oxidation of β-keto-acids in the liver. On the other hand, the rate of the molecular dissociation of choleic acids into their molecular constituents subsequent to solution may be estimated in these choleic acids by titration of the liberated enol form of the acholic constituent[1]. These measurements illustrate the stability of choleic acids from a different angle.

Stability of choleic acids. Several features in the behaviour of the bile acids towards lipoids and other water-insoluble substances are shared by the saponin group. But while many of these polycyclic substances enter into molecular compounds, for instance digitonin with cholesterol (1 : 1), the formation of molecular compounds of higher order seems to be limited to the desoxycholic and apocholic acids found in or derived from animal bile[2]. Whether this peculiarity signifies a specially high degree of affinity present in smaller measure in other bile acids, or whether it is an individual distinctive feature of bile acids with two hydroxyl groups, is not known; but it offers an approach to quantitative investigations on the influence of bile acids on lipoid metabolism. One must bear in mind however that these substances differ not only in their co-ordination numbers, but in their stabilities. Choleic acids of equal co-ordination number will vary through the volatility of the acholic constituent, through their solubility in various solvents, the rate of their molecular dissociation (see above), and through their stability in the presence of substances competing for co-ordinative linkage with the different bile acids.

Multiple proportions in co-ordination compounds. It is imaginable that two choleic acids differing in their co-ordination numbers are formed from one acholic constituent. This possibility has been exemplified by Rheinboldt, König and Flume [1929] in the case of camphor. We are able to add a few instances where choleic acids were formed in two proportions (Part III). The conditions for the formation of these compounds, their stabilities and their place in lipoid metabolism remain to be investigated.

II. CO-ORDINATION COMPOUNDS OF POLYMETHYLENE DICARBOXYLIC ACIDS WITH DESOXYCHOLIC ACID.

The general method of synthesis of molecular and dicarboxylic acid compounds with desoxycholic acid is as follows. Very pure desoxycholic acid and the acholic component are dissolved in a small amount of hot absolute ethyl alcohol. The crystals obtained upon cooling are separated and dried under

[1] The term *acholic* constituent is suggested for any chemical substance combining with a bile acid to form a choleic acid.

[2] Cholic acid crystallises with 1 molecule of alcohol.

reduced pressure in an Abderhalden pistol. The substance is then recrystallised from ethyl alcohol until it shows a constant composition. This method may be applied in all cases where the solubility of the desired choleic acid in alcohol is less than that of alcohol-choleic acid. It failed in the present series with glutaric and pimelic acids since the first crop of crystals consisted of a mixture of alcohol-choleic acid and the dicarboxylic acid-choleic acid. On subsequent recrystallisation pure alcohol-choleic acid was obtained. In all other instances however a synthetic product of constant composition was obtained.

Analysis of choleic acids. Choleic acids, synthesised from two known components, may be isolated free from solvent by properly instituted drying procedures. Nevertheless, elementary analysis does not yield conclusive evidence as to the proportional shares of the two components, owing to the small differences in elementary composition.

The xylene method of Wieland and Sorge [1916], extensively used by Rheinboldt [1926], is based on the dissociation of the aliphatic-choleic acid in xylene yielding the insoluble xylene di-choleic[1] acid *plus* the free aliphatic acid. The latter is subsequently isolated from its xylene solution and titrated. While Rheinboldt applied this procedure with advantage to the analysis of monocarboxylic acid-choleic acids, the insolubility of dicarboxylic acids in xylene creates a new difficulty and one must resort to a more involved method including the isolation of the calcium salt [Rheinboldt, König and Otten, 1929, p. 262].

Whenever the acholic constituent has either a low acid equivalent or no acid character at all, the acid equivalents computed for choleic acids with various co-ordinative ratios are sufficiently far from each other to allow a definite decision regarding this ratio. Since the critical acid equivalents in the most unfavourable case of the present series (octodecamethylenedicarboxylic acid-choleic acid) were more than 2 % removed from each other, the determination of acid equivalents was the method of choice.

Another method of ascertaining the molecular ratio in a co-ordinative compound is based on the melting-point diagram [Rheinboldt, 1925; Rheinboldt and Kircheisen, 1926; Pfeiffer, 1927]. Various mixtures of the two components are fused together and after complete homogeneity is attained, the melting-point is determined. In most instances, a maximum can be observed in the melting-point diagram, when a compound exists, but never when no compound is formed. The composition of the compound is given by the abscissa corresponding to this maximum. It is practical to plot as abscissae molecular percentages instead of percentages by weight.

This method offers independent corroboration of the analytical results. But the interpretation of the melting-point curve will fail in cases where it has no sharp maximum or none at all. It is necessary, then, to resort to the thawing-point curve [Auftau-Diagramm, Rheinboldt, 1925]. This curve is

[1] We propose the terms *di-choleic acid, tri-choleic acid, etc.*, to designate molecular compounds containing two, three, *etc.* molecules of desoxycholic acid.

constructed from the temperatures at which the mixture will start to liquefy, while the melting-point curve is defined by complete disappearance of the solid phase.

The substances A and B (Fig. 1) form a compound of the molecular ratio 1 : 2. There are five points where the melting-point curve and the thawing-point curve coincide, *i.e.* where a sharp melting-point is observed, the two pure components, their molecular compound, and the two eutectic mixtures $(A + AB_2)$ and $(AB_2 + B)$. With any other mixture, there is a greater or smaller range of temperature between thawing-point and melting-point, where a solid and a liquid phase coexist. Everything is liquid in the field above the melting-point curve and everything is solid below the thawing-point curve. The solid substance A coexists with the liquid mixture of variable composition in the wing-shaped field enclosed by the two curves leading from the melting-point of pure A to that of the eutectic $(A + AB_2)$. In the similarly shaped field between $(AB_2 + B)$ and pure B, solid B forms the solid phase of the biphasic mixture. The two fields above the central parts of the thawing-point curve and below the centre span of the melting-point curve symbolise the existence of solid AB_2 in a melt, containing an excess of A or B, for the temperature ranges given.

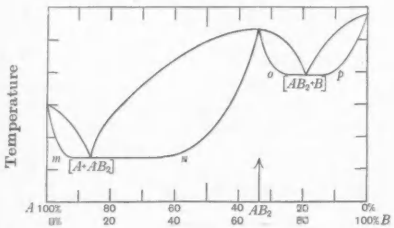

Fig. 1.

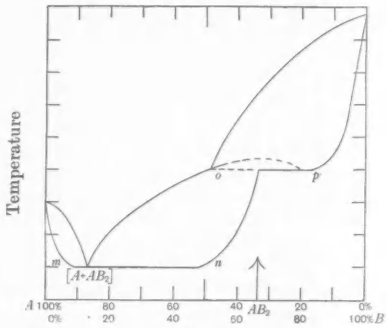

Fig. 2.

Fig. 1. Melting- and thawing-point diagram of a system $A + B$ with a homogeneously melting compound AB_2. (According to Rheinboldt.)

Fig. 2. System $A + B$ forming a compound AB_2 which does not melt homogeneously. (According to Rheinboldt.)

Diagrams of the general type represented by Fig. 1 are obtained in the case of many choleic acids, *e.g.* in Fig. 3 exemplifying the system sebacic acid + desoxycholic acid. A maximal melting point is found at 181° for a mixture of 20 mol. % sebacic acid + 80 mol. % desoxycholic acid. The compound AB_4 has a very wide range of existence and the melting-point curve reaches a flat but definite maximum. The eutectic mixtures approach the pure compounds so closely in composition that the melting-point curve cannot be verified experimentally for the branches A to $(A + AB_4)$ and $(AB_4 + B)$ to B. But the melting-points of the eutectic mixtures are revealed by the ordinates of the two horizontal branches of the thawing-point curve. These two horizontals are designated m–n and o–p in Figs. 1 and 2 and will be referred to as eutectic levels.

In some instances, curves of another type are obtained (Fig. 2). The maximum of the melting-point curve is hidden and the compound does not melt sharply and homogeneously, but the component with the lower melting-point melts at a faster rate, while the coexisting solid phase is enriched in substance B. Such a curve was observed with pimelic acid, one of the lower odd-numbered acids with which the preparation of pure choleic acids by recrystallisation failed. The co-ordinative affinity of these acids is so weak that the acholic constituent leaves the crystal structure and dissolves or melts without simultaneous liquefaction of the other partner, namely without complete disruption of the desoxycholic acid pattern. This interpretation is in effect the same as

that given above, when the failure to prepare certain choleic acids was explained by their greater solubility as compared to alcohol-choleic acid. An analysis of the thawing-point curve may be helpful for the determination of the co-ordination number in these instances.

The following variations and peculiarities were noted in the choleic acids studied.

(1) The melting-point of the molecular compound is higher than that of either constituent. This condition was encountered with sebacic acid (Fig. 3) and all higher acids studied in the series

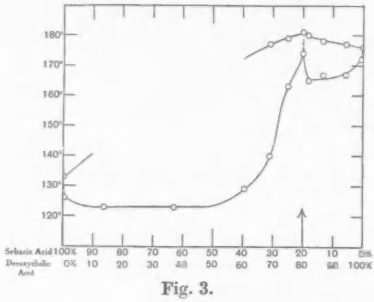

Fig. 3.

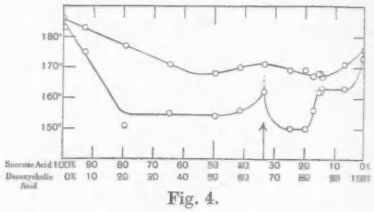

Fig. 4.

Fig. 3. System sebacic acid + desoxycholic acid.

Fig. 4. System succinic acid + desoxycholic acid.

(2) Both components melt at a higher temperature than their compound in the instance of succinic acid *plus* desoxycholic acid (Fig. 4). However, the compound has a definite maximum melting-point and the thawing-point of 162° with the same percentage composition is 8° above the higher one of the two eutectic levels (154° and 150°). A peculiar irregularity in the succinic acid-desoxycholic acid thawing-point curve remains to be explained. There is an indication of a third eutectic level between 15 and 7 % succinic acid at 163°, implying the existence of a second less stable molecular compound of the possible composition 1 : 6.

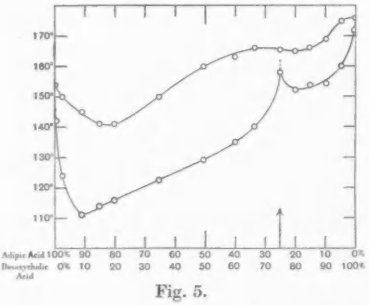

Fig. 5.

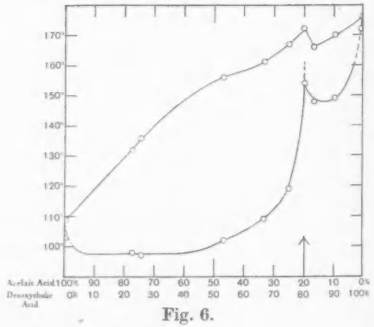

Fig. 6.

Fig. 5. System adipic acid + desoxycholic acid.

Fig. 6. System azelaic acid + desoxycholic acid.

(3) The melting-point of the compound is between the melting-points of the constituents as with adipic and azelaic acids (Figs. 5 and 6). This is also assumed in the example given in Fig. 1. While a definite turning point appears in the melting-point curve of azelaic acid at the composition 1 : 4, the adipic acid curve is almost horizontal between 33 and 15 % adipic acid. It is for the thawing-points only that a decision can be reached confirming the molecular ratio 1 : 3. The conditions in adipic acid-choleic acid resemble those illustrated in Fig. 2 and in pimelic acid-choleic acid.

In the case of pimelic acid (Fig. 7) the melting-point of the compound is situated between those of its components, but there is no initial decrease in the melting-point on the branch from the melting-point of the compound toward the higher-melting component. In any mixture containing an excess of the higher-melting component this component will be less soluble in the melt than the molecular compound. Solid *B* (desoxycholic acid) will coexist with the liquid phase throughout the triangular field enclosed by the eutectic horizontal *o–p* and the two curves leading upwards to the melting-point of *B*. The melting curve of pimelic acid-choleic acid conforms closely with the schematic case of Fig. 2. The thawing curve at 20 and 17·5 % pimelic acid (molecular ratio 1 : 4 and 1 : 4·7) shows an elevation over the lower thawing-point 131° at 14·7 % pimelic acid which represents the eutectic level *o–p*. It is doubtful whether deviations from the theory as in this instance, and in the case of succinic acid discussed above, are due to experimental errors, impurities and the like. We are inclined to believe that these exceptions are caused by the interference of molecular compounds of different composition and lower stability. These compounds are completely dissociated at melting-temperature but they may play a rôle at lower temperatures thus causing the complications mentioned. Such interferences are more apt to be displayed by molecular compounds of higher order like choleic acids than by the molecular compounds with the ratio

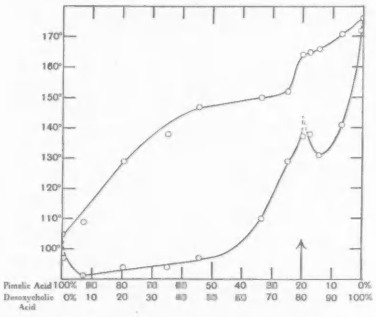

Fig. 7. System pimelic acid + desoxycholic acid.

1 : 1 or 1 : 2 which formed the basis for the development of the melting-point and thawing-point theories. The application of Weissenberger's studies [1924, 1925] on depression of vapour tension in molecular compounds to choleic acids with volatile acholic components might aid in the elucidation of this point.

As mentioned previously, the range of existence of the choleic compounds commonly approaches so closely the 0 and 100 % ordinates that the minimum melting-points of the two eutectic mixtures ($A + AB_n$) and ($AB_n + B$) cannot be verified. A perusal of Rheinboldt's experiments [1926, 1928, 1929] shows that this holds for all choleic acids investigated excepting the compounds of camphor with desoxycholic and apocholic acids with the molecular ratio 1 : 1. This is in contrast with the numerous systems like picric acid + naphthalene where all features demanded by the theory may be verified [Rheinboldt, 1925]. But even when the melting-point curve shows the theoretical drop, with increasing percentages of bile acid being added to the pure fatty acid, as in the cases of succinic and adipic acids, the low inflection points of the melting-point curves at 168° with 50 % succinic acid and at 141° with 85 and 81 % adipic acid are so far from the thawing-point curves as to exclude the interpolation of sharply-melting eutectic mixtures. Here too the deviation from the theoretical course seems to be more deeply rooted than in experimental fallacies. It may be noted that no eutectic level but a straight diagonal runs from 110° to 140° in the thawing diagram of adipic acid. A slight slant of the *m–n* branch obtains also with pimelic acid, while all the other systems studied lack this irregularity.

Table I is a synopsis of these results and the findings by the acid equivalent titrations. The melting-point of the acholic component is given for comparison

with the melting-point of the choleic acids. Except in the case of pimelic acid, all co-ordination numbers were confirmed independently by the estimation of the acid equivalent. We confined ourselves to the latter method in the case of

Table I. *Melting-points, acid equivalents and co-ordination numbers of dicarboxylic acid-choleic acids.*

Acid	Formula	Melting-point °C. Free acid	Melting-point °C. Choleic acid	Acid equivalent Found	Acid equivalent Calculated (N − 1)	Acid equivalent Calculated N	Acid equivalent Calculated (N + 1)	Co-ordination number N
Succinic	$CO_2H(CH_2)_2CO_2H$	186	171	228	170·1	225·7	259·0	2
Adipic	$CO_2H(CH_2)_4CO_2H$	154	165·5	271·5	232·7	264·6	285·2	3
Pimelic	$CO_2H(CH_2)_5CO_2H$	105	—	—	—	—	—	4
Suberic	$CO_2H(CH_2)_6CO_2H$	142	172·5	285·5	270·2	289·9	304·5	4
Azelaic	$CO_2H(CH_2)_7CO_2H$	109	172	296·5	273·0	292·2	306·5	4
Sebacic	$CO_2H(CH_2)_8CO_2H$	133	181	294	275·8	295·2	308·5	4*
	$CO_2H(CH_2)_9CO_2H$	111	172	299	278·6	297·6	311·1	4
	$CO_2H(CH_2)_{10}CO_2H$	129	183·5	322	312·5	323·0	330·7	6
Brassylic	$CO_2H(CH_2)_{11}CO_2H$	116	181	323·5	314·5	324·8	332·2	6
	$CO_2H(CH_2)_{12}CO_2H$	126	186·5	326	316·5	326·5	333·8	6
Thapsic	$CO_2H(CH_2)_{14}CO_2H$	125	188	332	320·5	330·0	336·9	6
	$CO_2H(CH_2)_{16}CO_2H$	125·5	191·5	333·5	324·6	333·5	340·1	6
	$CO_2H(CH_2)_{18}CO_2H$	126·5	192	335	327·1	335·3	341·6	6

* *Cf.* Wieland [1928].

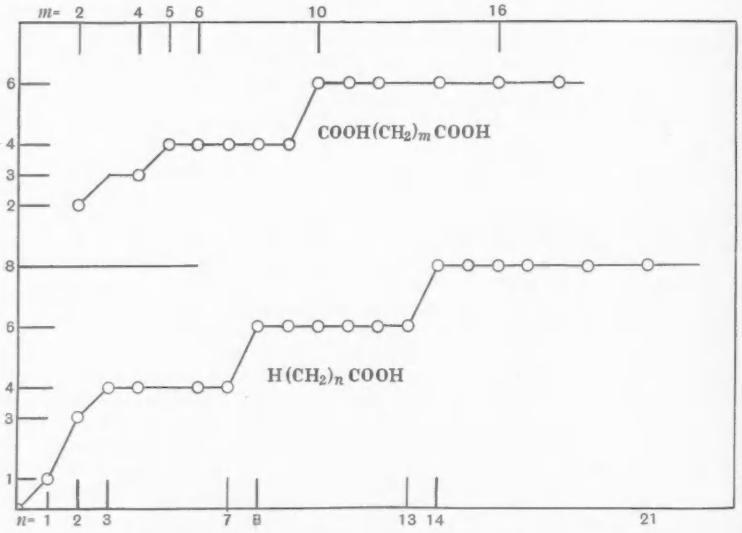

Fig. 8. Relation between length of aliphatic chain and co-ordination number.

suberic acid, enneamethylenedicarboxylic acid and the four acids with high molecular weight. While the melting-point diagram allowed no decision for pimelic- and adipic-choleic acids, the ratio 1 : 3 could be ascertained for the latter both by the thawing-point diagram and the acid equivalent. Preference

was given to the ratio 1 : 4 over 1 : 5 for pimelic acid-choleic acid for the theoretical reason that 5 never occurs as co-ordination number and that the homologues following pimelic acid combine definitely in the ratio 1 : 4 with bile acid.

Fig. 8 gives a graphic representation of the correlation between number of methylene groups and co-ordination number in the present series. The co-ordination number for glutaric acid is inserted arbitrarily with little hope that a glutaric acid-choleic acid may be synthesised, as preliminary experiments showed that such a compound must be extremely labile. The analogous terrace-shaped diagram given by Rheinboldt [1926, 1929] and based on his own and some of Wieland's data is added beneath to facilitate comparison.

Possible relations between these periodic changes in co-ordinative valency and biochemical behaviour were discussed in Part I.

Experimental.

Source of material. The following acids of the polymethylenedicarboxylic series were synthesised. Enneamethylenedicarboxylic acid, decamethylenedicarboxylic acid and hexadecamethylenedicarboxylic acid were prepared according to Chuit [1926] by reduction of diethyl azelate, diethyl sebacate and diethyl thapsate with sodium in alcoholic solution following Bouveault's procedure. The resulting diols were converted into 1 : 9-dibromononane, 1 : 10-dibromodecane and 1 : 16-dibromohexadecane respectively and subsequently into the corresponding dinitriles which were saponified by alcoholic alkali to yield the dicarboxylic acids. Dodecamethylenedicarboxylic acid and octadecamethylenedicarboxylic acid were prepared from 1 : 10-dibromodecane and 1 : 16-dibromohexadecane respectively with ethyl sodiomalonate and saponification followed by partial decarboxylation of the resulting dodecane (or octadecane)-tetracarboxylic acid. Pimelic acid was prepared by reduction of salicylic acid with sodium in amyl alcohol [Einhorn and Willstätter, 1894; Einhorn and Lumsden, 1894]. We are indebted to the firm M. Naef et Cie., Geneva, for a specimen of brassylic acid. Some of the commercial acids were purified by vacuum distillation of the diethyl ester or of the free acid itself.

Pure desoxycholic acid was prepared from acetic acid-choleic acid or from commercial desoxycholic acid in the following manner. 100 g. of the starting material which should be strictly free from cholic acid are dissolved in 500 cc. hot ethyl alcohol. The filtered solution is concentrated to less than 200 cc. on an electric hot-plate and the syrupy residue poured with constant stirring into 1500 cc. of cold ether. A fine sandy powder settles within a few minutes and is filtered off with suction. If the amount of alcohol poured into the ether has been kept low, only a few grams of bile acid will be left in the ethereal filtrate at this stage. The ether retains all lipin impurities. The crystalline ether-choleic acid is dissolved in one or two parts of absolute alcohol and recrystallised. At this stage, alcohol-choleic acid should be obtained in long transparent prisms. They are carefully separated from the mother-liquor and may be recrystallised once more to show a melting-point of about 118°–120°. These procedures should not involve a loss of more than 40 g.

This alcohol-choleic acid is sufficiently pure for syntheses in alcoholic solution. For the determination of thawing-points and melting-points it is

essential to provide the purest desoxycholic acid. This is obtained from the pure alcohol-choleic acid of which 60 g. are taken up in 100 cc. of alcohol and poured into a wide beaker containing 1 litre of boiling water. Boiling is maintained for several hours, water being added to replace losses by evaporation [*cf.* White, 1929, p. 1171]. The pasty white mass changes into heavy hard lumps as alcohol is given off. These lumps are separated, roughly dried, ground and finally dried to constant weight. The melting-point of this product was 176° (corrected, see below), its thawing-point was 171–172°. Its purity was further checked by its optical activity.

It is worth noting that various lots of alcohol-choleic acid differ in the tenacity with which the alcohol is retained under reduced pressure and high temperature. On the other hand, it was observed that desoxycholic acid, free from solvents, would increase in weight slightly when kept for a few months in a desiccator over phosphorus pentoxide, simultaneously its thawing-point would drop below 170°. Therefore, free desoxycholic acid used in this work was always freshly prepared from a stock of alcohol-choleic acid.

Syntheses of choleic acids. As a rule, 2 g. of the dicarboxylic acid were added to a warm solution of 8 g. desoxycholic acid in 20–30 cc. of alcohol. The choleic acid separated on cooling at varying speed as a crystalline powder. This was separated and dried in an Abderhalden drying apparatus at 80° in a high vacuum, until a weighed specimen had reached constant weight. Its melting-point and its acid equivalent were then determined. A portion of the specimen was recrystallised from ethyl alcohol and these procedures were repeated two or three times when necessary. In several instances the first or second fraction showed a higher melting-point than the subsequent ones. This holds particularly for the lower members of the series. As discussed in the theoretical part, these choleic acids are less stable and will crystallise in presence of an excess of the acholic component only. When recrystallisation is attempted from alcohol or other solvents, the solvent-choleic acid gradually replaces the desired choleic acid, thus leading to products of lower melting-point and higher acid equivalent approaching, with alcohol as solvent, 438 (ethyl alcohol-choleic acid). Even less labile choleic acids cannot be recrystallised except within a certain limit of excess of either constituent. The crystal fraction with the highest melting-point was used for the determination of the acid equivalent.

Determination of acid equivalents. The acid equivalents were determined by dissolving 50–100 mg. of substance in 4–5 cc. of methyl alcohol and titrating with 0·05 *N* aqueous NaOH with bromothymol blue as indicator. In some instances an excess of alkali was used and titrated with 0·05 *N* HCl. Both methods yield identical values. For the calculation of the acid equivalents, the mol. wt. of desoxycholic acid was assumed to be 392·32, *e.g.* the acid equivalent for sebacic acid-tetracholeic acid is obtained from

$$\frac{(392{\cdot}32 \times 4) + (202{\cdot}14 \times 1)}{4+2} = 295{\cdot}2.$$

The results of these analyses are summarised in the theoretical part.

Melting- and thawing-points. The mixtures of varying molecular composition are prepared as follows. 5 mg. or more of the acholic component are weighed with an accuracy of 0·01 mg., if possible on a micro-balance, into a glass thimble made from a light test-tube and measuring about 12 mm. in diameter and 20 mm. in height. An amount of desoxycholic acid is added approximating as closely as possible to the amount computed for the intended molecular ratio. The thimble is placed on a hot-plate in a 30 cc. beaker which serves as an air-bath; 2–3 drops of methyl alcohol are added. This solvent was chosen because it is less firmly held *in vacuo* than ethyl alcohol or acetone. The mixture dissolves in the methyl alcohol and while homogeneity is attained part of the solvent evaporates. Great care has to be exercised to avoid spattering even for a few moments after removal from the hot-plate. As many as four thimbles may be carefully introduced at the same time into a double-jacketed Abderhalden pistol and dried for 1 hour at 80° in the case of dicarboxylic acid-choleic acids. After cooling, the scaly mixture is broken up and triturated with a heavy rounded rod serving as a pestle.

For the observation of the thawing- and melting-points, we use a Thiele-Anthes melting-point flask [Rheinboldt, 1925]. The sulphuric acid is agitated by a current of dried air. The thermometer is calibrated in the melting-point apparatus used against a set of Anschütz thermometers which are immersed above the meniscus. In this manner corrected melting-points are obtainable. As the corrections in the essential range of temperature reach 3–4° a uniform correction is very desirable.

The substance is tightly packed in a column 6–8 mm. high by means of a fine glass rod in the melting-point tubes, which should not be too narrow. The melting-point tube is placed so that its tip is situated in front of and in contact with the bulb of the thermometer. The substance is observed through a magnifying glass. The melting-point tube may be rotated for close scrutiny at the upper end protruding through a side-tube of the Thiele apparatus. The temperature is raised at a rate not exceeding 5° per minute but at the critical temperatures a considerably lower rate will be found advantageous. While one observer watches the substance, a second observer follows the rise of the mercury on the thermometer scale which is turned at a right angle from the first observer. The second observer records the observations made by the first one.

The thawing-point of a mixture is defined by its incipient liquefaction. If thorough homogeneity of the sample is attained, the beginning of liquefaction is manifested by the simultaneous appearance of droplets at many points on the walls of the melting-point tube. These droplets coalesce, and usually within a few degrees rise the crystalline powder will lose its lustre. This transition is described by Rheinboldt [1925] as a change from a silky to a velvet-like appearance and we found that this simile is very apt to describe the phenomenon in many cases. At this point, the substance will shrink, giving rise to horizontal fissures. After this, the mass changes into a few drops separated by air bubbles. A few degrees hence will suffice for the substance to reach its melting-point.

The opaque fluid clears, starting at the periphery and from the bottom, but it should not be pronounced melted until the last traces of a crystal skeleton have disappeared in the centre or towards the interfaces of the air bubbles. Care should be taken not to confuse these last crystals with foam occluded in the viscous fluid.

SUMMARY.

The synthesis and analysis of choleic acids is discussed and exemplified by the molecular compounds of desoxycholic acid with aliphatic dicarboxylic acids. The co-ordinative valences of these acids were ascertained by the determination of the acid equivalents of the choleic acids and by a study of their melting- and thawing-point diagrams. The theory of these diagrams is discussed, deviations are enumerated, and their explanation is attempted.

The co-ordination number of succinic acid is 2, of adipic acid 3, of pimelic, suberic, azelaic, sebacic and enneamethylenedicarboxylic acids 4, and that of decamethylenedicarboxylic, brassylic, dodecamethylenedicarboxylic, thapsic, hexadecamethylene- and octadecamethylene-dicarboxylic acids 6.

III. STRUCTURAL ISOMERISM AND CO-ORDINATIVE VALENCY. MULTIPLE CO-ORDINATION NUMBERS.

This part deals with the relationship between chain-isomerism and co-ordination number in the desoxycholic acid compounds of the valeric and the butyric acids.

The choleic acids of these acids were prepared by direct synthesis, *e.g.* 5 g. of pure desoxycholic acid were dissolved in 20 cc. of the aliphatic acid and kept at room temperature or in the ice-chest. A crop of crystals separated and was dried *in vacuo* over KOH until constant weight and constant acid equivalent was reached. The other method of synthesis, namely in alcoholic solution (see Part II, p. 564), led to the formation of choleic acids in the case of *n*-butyric and *n*-valeric acids only. The branched isomerides, and in addition propionic acid, fail to form choleic acids in alcoholic solution, apparently because of the less stability and greater solubility of their molecular compounds, as was previously discussed in the case of glutaric acid. In every one of these instances alcohol-choleic acid was obtained as the main product.

The co-ordination number of all these choleic acids was found to be 2 except of *n*-butyric and *n*-valeric acids which combined with 4 desoxycholic acid molecules in alcoholic solution in agreement with Wieland and Sorge's results. However, a *n*-butyric-dicholeic acid was obtained by direct synthesis. Thus, two choleic acids differing from each other in their co-ordination numbers 2 and 4, are formed from butyric acid, as in the case of camphor (1 and 2).

Tiglic, sorbic and oleic acids display the same co-ordinative valency as the saturated acids of the same number of carbon atoms. Crotonic acid, however, combined with desoxycholic acid in the molecular ratio 3 : 1 while the maximal

co-ordination number of butyric acid is 4. The influence of the double bond on co-ordinative valency is greater in a small molecule and it seems to alter the character of configuration. Crystallographic measurements and especially crystal analysis by X-rays [Herzog, Kratky and Kuriyama, 1931] will increase our understanding of these conditions.

A third method for the synthesis of choleic acids consists of the addition of a water-insoluble acholic constituent to an aqueous solution of sodium desoxycholate. In the case of ethyl butyrate, the thin layer of ester placed on top of the bile salt solution solidifies within a short time. The needle-shaped crystals isolated from this layer consist of ethyl butyrate-choleic acid. It is noteworthy that the sodium ion is left behind in the aqueous phase. This is an instance of hydrolytic adsorption where the contact with a non-electrolyte phase alters the concentration of the electrolytes of the aqueous phase and eventually influences the p_H. Such processes have a bearing on the p_H-adjustment of bile which is being studied in this laboratory [Ottenberg and Kahn, 1932].

Ethyl butyrate-choleic acid prepared in this manner shows a molecular ratio 4 : 1 in agreement with Rheinboldt's rules for the co-ordination number of esters. A second ethyl butyrate-choleic acid may be obtained from ethyl butyrate *plus* desoxycholic acid in alcoholic solution. This substance contains 2 molecules bile acid for each molecule of ester.

Table II. *Acid equivalents and co-ordination numbers of choleic acids.*

	Acid equivalent					
		Calculated			Co-ordination number	
Acid	Found	(N − 1)	N	(N + 1)	N	Method of synthesis
Propionic	288	233·2	286·2	312·8	2	Direct
n-Butyric	335	316·3	331·5	341·5	4	Alcohol
	292	240·2	290·9	316·3	2	Direct
*iso*Butyric	296	240·2	290·9	316·3	2	Direct
n-Valeric	329	319·8	334·3	343·9	4	Alcohol or direct
*iso*Valeric	294	247·2	295·6	319·8	2	Direct
Methylethylacetic	296	247·2	295·6	319·8	2	Direct
Trimethylacetic	297	247·2	295·6	319·8	2	Direct
Crotonic	313	290·2	315·8	331·1	3	Alcohol
Ethyl butyrate	452	508·4	450·4	431·0	2	Na-desoxych.
	420	431·0	421·3	415·5	4	Alcohol

None of the choleic acids mentioned above shows a definite melting-point, as they all gradually lose their acholic constituent between 140° and 150°. None the less, they are definite chemical entities, as they are stable and of constant acid equivalent at normal temperature and pressure and even during prolonged storage in a desiccator over alkali hydroxide. They are odourless despite the great volatility and strong odour of their acholic constituents.

The following experiment illustrates the influence of temperature on the co-ordination number. 1149 mg. ethyl alcohol-choleic acid (molecular ratio 1 : 1) contains 120·5 mg. alcohol. When heated in an Abderhalden drying apparatus at 80° *in vacuo*, it lost 64 mg. (56 % of the alcohol) within 15 minutes.

The other half of the alcohol was tenaciously retained and the additional loss after 4 hours under the same conditions was 7·5 mg. only. When heated at 95° for 15 and 30 minutes and at 110° for 1 hour the losses were 55·5 % and 62 %. The product obtained by heating alcohol-choleic acid *in vacuo* to 80–100° has therefore the composition of an alcohol-dicholeic acid. Mörner [1924] investigated the stability of acetic acid-choleic acid at normal temperature and pressure over H_2SO_4 and CaO. The volatilisation of the acholic component in this choleic acid proceeds gradually without perceptible sudden retardation after the loss of one-half or any other fraction of its percentage of acetic acid.

Summary.

The co-ordination number of isomeric butyric and valeric acids is 2 with the exception of *n*-butyric and *n*-valeric acids which display a maximal co-ordinative valency of 4.

Butyric acid and ethyl butyrate form two choleic acids each, depending on the method of synthesis with molecular ratios 4 : 1 and 2 : 1. A second ethyl alcohol-choleic acid of molecular ratio 2 : 1 is formed at elevated temperatures.

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LXVIII. THE IODIMETRIC METHOD OF DETERMINING LACTOSE IN MILK.

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Iodimetric determination of an aldehyde was first carried out by Romijn [1897], who utilised the oxidation of formaldehyde to formic acid by iodine in alkaline solution for this purpose. The application of the method to aldoses was investigated by Willstätter and Schudel [1918], who found that reaction proceeded quantitatively only when (1) the iodine added was twice the amount required theoretically; (2) the alkali was $1\frac{1}{2}$ times the equivalent of the iodine added; (3) the reaction was terminated in 15–20 minutes.

The quantitative conditions have been confirmed by subsequent workers, but there has been disagreement as to the time required to complete the reaction. Willstätter and Schudel's data were with reference to aldoses in a concentration of 1 %: Judd [1920] also found 15–20 minutes necessary for the reaction, but Baker and Hulton [1920] found that 5 minutes sufficed.

My own findings are that 5 minutes suffice for 0·5 to 1 % solutions of aldoses, whilst 20 minutes are required for 0·1 % solutions. Macleod and Robison [1929] obtained a similar result for 0·1 % aldose solutions.

Application to lactose in milk.

This was first carried out by Kolthoff [1923], who clarified the milk with freshly precipitated zinc ferrocyanide. The choice of a suitable clarifying agent is a matter of great importance in the iodimetric method, and I have found the ordinary precipitants (alumina cream, acid mercuric nitrate) unsuitable owing to fluctuations caused in the results by the presence of excess reagent in the filtrate. I finally adopted "colloidal iron" (B.D.H.), added in the ratio of 7 cc. to 10 cc. milk. It was added drop by drop from a burette, with constant stirring of the milk. The resulting mixture filtered rapidly through coarse filter-paper (Chardin), and contained no inorganic constituent derived from the clarifying reagent.

Accuracy of iodimetric method.

(1) *Comparison with gravimetric copper method.* Using the O'Sullivan [1909] modification of the gravimetric estimation of lactose the following results were obtained.

No. of sample milk	% lactose	
	Iodimetric	Gravimetric
1	5·09	5·10
2	5·13	5·19

(2) *Comparison with polarimetric method.* Kolthoff found the iodimetric method to give higher results than the polarimetric. This discrepancy is usually assumed to be due to non-sugar substances in milk-serum reacting with iodine and giving too high a result. This assumption has not been proved, and it is interesting that a similar discrepancy between polarimetric and gravimetric methods in the case of human milk lactose has been recently explained by the presence of two aldoses other than lactose, *i.e.* gynolactose (laevorotatory) and allolactose (dextrorotatory). The presence of these sugars would clearly produce a higher result by reduction methods than by optical methods [Polnovski, Michel and Lespagnol, 1931].

(3) *Comparison with chloramine-T method.* The chloramine-T method has been fully investigated by Hinton and Macara [1927], and has been favourably commented on by the Milk-Products Sub-Committee to the Standing Committee on uniformity of analytical methods [1930].

Hinton and Macara considered their method superior to the iodimetric one, which was stated to be less accurate because (1) it is more difficult to control owing to the rapidity of the reaction (5 minutes as compared with 1–1½ hours for the chloramine-T method); (2) non-sugar substances are more liable to be oxidised by the alkaline iodine solution.

No comparative figures were given to substantiate these criticisms and my own conclusions, based on the following results, show that the two methods agree very closely, and that any oxidation of non-sugars occurs in the chloramine-T rather than the iodimetric method.

In comparing the two methods the following techniques were adopted.

Iodimetric method. 50 cc. milk in a litre beaker are diluted with 250 cc. distilled water and treated from a burette with 35 cc. "colloidal iron," at the rate of a few drops a second, mixing the contents constantly by a whirling movement; 165 cc. distilled water are added and the mixture filtered through coarse filter-paper. The filtrate is a 10 % milk-serum.

To 25 cc. of the filtrate in a conical flask are added 25 cc. $N/10$ iodine and 37·5 cc. $N/10$ NaOH. The solution is shaken and after 5 minutes treated with 37·5 cc. $N/10$ H_2SO_4 and titrated with $N/20$ sodium thiosulphate.

25 cc. $N/10$ iodine are titrated with $N/20$ sodium thiosulphate. If the difference between the two titrations $= X$ then % lactose in milk-serum $= 0{\cdot}036\ X$ (% in milk $= 0{\cdot}36\ X$).

Chloramine-T method. To 25 cc. milk-serum prepared as above are added 3 cc. NaOH, 20 cc. 10 % KI and finally 50 cc. $N/20$ chloramine-T: after 1½ hours the solution is treated with 10 cc. $2N$ HCl and titrated with $N/20$ sodium thiosulphate.

Similar reagents are added to a control flask containing 25 cc. distilled

water. After 1½ hours the solution is acidified and titrated as before. If the difference between the two titrations = X cc. then lactose % = 0·036 X.

No. of milk sample	% lactose	
	Iodimetric method	Chloramine-T
1	4·95	4·97
2	5·00	4·99
3	5·22	5·27
4	5·25	5·28
5	5·27	5·33
6	5·06	5·15
7	5·30	5·40
8	5·20	5·24
1 g. lactose added to 100 cc. of sample 8	6·20	6·30

Range of accuracy of iodimetric method. 1 % hydrated lactose solution was accurately prepared and varying quantities were taken from 5 cc. up to 25 cc., distilled water being added in each case to make the volume up to 25 cc.

The solutions were then analysed for lactose by the method already described, and the following results obtained:

Vol. of 1 % lactose taken (cc.)	Water added (cc.)	cc. $N/20$ iodine absorbed in 5 mins. (X)	% lactose 0·036 X	Theoretical result	Error (%)
25·0	0·0	26·50	0·954	1·0	−4·6
22·5	2·5	24·35	0·877	0·9	−2·6
20·0	5·0	22·00	0·792	0·8	−1·0
17·5	7·5	19·25	0·693	0·7	−1·0
15·0	10·0	16·60	0·598	0·6	−0·3
12·5	12·5	14·00	0·504	0·5	+0·8
10·0	15·0	11·15	0·401	0·4	+0·3
7·5	17·5	8·40	0·302	0·3	+0·7
5·0	20·0	5·70	0·205	0·2	+2·5
0·0	25·0	0·00	—	—	—

Hence between the limits of 0·3 % and 0·8 % lactose there is a maximum error of 1 %. Milk-lactose has a value ranging from 4 % to 6 %, and therefore the 10 % serum obtained from milk usually varies between 0·4 % and 0·6 % in its lactose content. Hence the iodimetric method is admirably adapted to the estimation of lactose in milk when a maximum error of 1 % is not objected to. When a result exceeding 8 % is obtained, the 10 % serum should be diluted with an equal volume of water and the resulting 5 % serum analysed.

Conclusions.

1. The iodimetric method of estimating lactose in milk gives results closely agreeing with the gravimetric copper method.

2. It is at least as accurate as the chloramine-T method, and is much more convenient when a rapid estimation is required, the reaction taking 5 minutes as against 1½ hours in the chloramine-T method.

3. "Colloidal iron" is the best clarifying agent to use for the iodimetric method.

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LXIX. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

VII. THE NATURE OF THE RESIDUAL HEMICELLULOSES OF ROTTED STRAW.

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In the first paper of this series [Norman, 1929, 1] a detailed method was described for the evaluation of the furfuraldehyde-yielding constituents of plant materials, and the nature of these substances in straws was determined. Later, by employing these methods on decomposing straws, the loss of such constituents was followed and their rôle in decomposition investigated [Norman, 1929, 2].

The direct estimation of hemicelluloses has not yet been satisfactorily achieved owing to their heterogeneous structure, and accurate evidence of their removal as a whole was not possible. It was only possible to follow with certainty the changes in the content of pentose and uronic acid units. The hexose units present are indeterminable directly. Acid hydrolysis methods, such as have been proposed by Waksman and Stevens [1930], are unsatisfactory and erratic, inasmuch as it has been shown by Link and Niemann [1930] that the uronic acids are unstable in presence of the concentration of hydrolysing agent employed and undergo decarboxylation with the formation of reducing substances of unknown constitution.

As far as it was possible to judge from the loss of the pentose units in the hemicelluloses, these substances undergo a very rapid and extensive early loss in the decomposition of such materials as oat or rye straw. It seemed that about 50 % of the total hemicelluloses was fermented away in the first week, but that after this point, while the cellulose was being rapidly removed, the hemicelluloses suffered only a small but steady additional loss up to the end of the two months, which was the period of the experiment. Analyses of well-rotted materials which have been made at other times have indicated that when decomposition is practically at a standstill only traces of hemicelluloses remain.

The hemicelluloses are a very indefinite class of polysaccharides, and their physical properties are such that no separation is entirely satisfactory. Hydrolysis studies suggest that the type of linkage involved is similar in different

fractions, and in hemicelluloses from different sources. Biological availability, however, does not depend only on linkage, but also on the nature of the units involved. The following sugars are commonly found—arabinose, xylose, glucose, galactose and mannose, together with glycuronic and galacturonic acids. These vary very much in their utilisation by micro-organisms, so that there is the possibility that certain groups may be preferentially removed, leaving intermediate residues which are attacked more slowly. In the studies already described, the losses of uronic acid from the hemicelluloses of oat straw were very different from those of the pentose units, while in the rye straw series they were rather similar. Attention was not drawn to this at the time. In view of the variations in availability between the constituent units of hemicelluloses it was thought possible that resistant groupings might remain, even at the close of an active fermentation, and an investigation was accordingly made of the nature and amount of the residual hemicellulose material of oat straw rotted under optimum conditions for many months.

Experimental.

One kg. of dry oat straw, well chaffed, was thoroughly moistened by spraying and placed in a large bottle to which available nitrogen in the form of ammonium carbonate was added at the rate of 1·0 g. N to 100 g. straw. The bottle was incubated at 35° and frequently turned to secure even distribution of the nitrogen and thorough and uniform wetting. From time to time additional water was added from a spray, water-logging, however, being avoided. Rapid decomposition set in, and the straw shrank in bulk and darkened in colour. By the end of nine months the straw had rotted to a black pulpy mass, without cellular structure. A portion was taken for analysis and the remainder extracted with 1500 cc. of water on a water-bath for 4 hours. The residue after filtration was re-extracted twice with 1000 cc. water for the same period. The combined filtrates were concentrated under reduced pressure and poured into three volumes of alcohol, yielding a light brown precipitate (precipitate I). The dark residue was then extracted with 1500 cc. of alcoholic sodium hydroxide (2 % NaOH in 60 % C_2H_5OH) for 4 hours. Two further treatments with alcoholic sodium hydroxide followed. The very dark filtrates obtained were combined, nearly neutralised with acetic acid, and concentrated under reduced pressure. When of small volume acetic acid was added, and a dark heavy precipitate was obtained (precipitate II). The residual straw was then treated twice with 500 cc. 2 % NaOH for 4 hours at 80°, followed by 4 % NaOH at 100° for 4 hours. The residue was washed many times with hot water, and dried. The combined alkaline extracts were made definitely acid with glacial acetic acid, whereupon a precipitate, grey-brown in colour, was obtained and was centrifuged out (precipitate III). To the acid-aqueous residue were added 2½ volumes of alcohol, and the precipitate obtained was filtered off on fluted filter-papers (precipitate IV).

Purification of the fractions. Precipitate I was redissolved in water and reprecipitated first by acid alcohol and then by neutral alcohol, finally being dried in increasing concentrations of alcohol, and over P_2O_5 in a vacuum desiccator; yield $<0{\cdot}5$ g. Precipitate II was washed several times with warm water and ether, and dried in a vacuum-oven; yield 8 g. Both precipitates III and IV were redissolved in alkali and precipitated by the addition of acid alcohol, finally being washed with alcohol and dried with increasing concentrations; yields, precipitate III $<1{\cdot}0$ g.; precipitate IV $<0{\cdot}5$ g.

The composition of the fractions. Owing to the smallness of the quantities available, the number of estimations which could be made was of course very limited. The pentose and uronic acid contents of the fractions were pre-eminently of interest and were determined in each case. Tests were made for mucic acid after oxidation with nitric acid, and, in the case of precipitate III, for arabinose by heating with diphenylhydrazine, in which case the result was positive.

The following results were obtained.

Precipitate	Ash %	Furfuraldehyde yield %	CO_2 yield %	Mucic acid
I	*	5·94	8·33	–
II	*	–	Trace	–
III	1·74	17·08	3·73	+
IV	*	12·38	3·12	+

* Not determined.

Discussion.

The water-soluble precipitate I from the rotted material bore no obvious relationship to any original constituent of the straw. The fact that on oxidation it yielded no trace of mucic acid precludes any possibility of relationship to pectin. It seems probable that this substance represents a microbial product akin to the bacterial gums. Hopkins, Peterson and Fred [1930, 1931] have recently reported that the water-soluble gum produced by strains of *Rhizobium* is a polysaccharide consisting of glucose and glycuronic acid. An osazone prepared from a very small quantity of the hydrolysed precipitate I resembled glucosazone; but the quantity was insufficient for characterisation and too much stress is not put on this point. The analytical figures indicate the presence of 33·3 % uronic acid anhydride, which accounts for 5·55 % furfuraldehyde. The balance of the furfuraldehyde, 0·4 %, might be due to pentose groupings or more probably to experimental error. It seems therefore that this substance contains about 66 % hexosan and 33 % uronic acid anhydride.

Precipitate II was found to yield no furfuraldehyde or CO_2 on treatment with HCl. Further it did not give reducing sugars on hydrolysis with dilute acid. This fraction probably contains lignin and "humic" matter, possibly the "hymatomelanic acid" described by Odén [1919]. It was not investigated further since it appeared not to be of polysaccharide nature.

Precipitates III and IV may be considered together. They are the fractions prepared in the same way as the A and B hemicellulose fractions of the straw, and may be compared with them.

	CO_2 yield %	Uronic acid anhydride %	Total furfur-aldehyde %	Pentose furfur-aldehyde %	Anhydro-pentose calculated as anhydro-arabinose %	Anhydro-hexose by difference %
Precipitate III	3·8	15·2	17·4	14·9	27·6	57·2
Hemicellulose A	2·7	10·8	42·9	41·3	76·7	12·5
Precipitate IV	3·1	12·4	12·4	10·3	19·2	68·4
Hemicellulose B	7·9	31·8	43·3	38·0	68·0	—

The calculations of anhydro-hexose are made with some reserve, inasmuch as there is little evidence of the purity of fractions obtained in such small quantities. It is possible that they might be contaminated with substances of a humic nature, possessing similar properties, though it is likely that soluble substances of this class would be removed by the pre-treatment with alkali-alcohol. From the positive mucic acid tests the presence of galactose or galacturonic acid in both fractions may be inferred. In both cases the residual products were much lower in pentose content than the straw products. It is probably safe to conclude that these substances represent small unavailable fragments of the original hemicelluloses, rather than elaborated products, since no water-insoluble microbial constituents or products which contain uronic acids and mixed sugars are known. This investigation failed to reveal the presence of any considerable amount of biologically unavailable hemicellulose, or of any resistant grouping formed as a consequence of incomplete attack. Instead it emphasised the complete availability of the hemicelluloses, an availability quite remarkable. The kg. of dry straw originally contained about 230 g. of free hemicelluloses, of which, at the close of the fermentation less than 2 g. could be recovered. It provides clear evidence that the structural arrangement and architecture of the hemicelluloses in the cell-wall is not such that the attack of micro-organisms is in any way hindered or retarded, as it is in the case of cellulose, by the presence of a more resistant lignin barrier. The cellulose in the same period suffered a loss of only two-thirds.

Variations in the biological availability of the hemicelluloses, or of units or constituent groupings in them, cannot therefore be of much importance in the case of the decomposition of straws by a general mixed flora, though it is likely that in pure culture decompositions they would be more obvious. In the earlier stages of decomposition by a mixed flora it is possible that such differences might be detectable. Information on this point can only be obtained by the laborious method of preparation of the hemicellulose fractions at different periods during the decomposition.

SUMMARY.

1. The nature of the residual hemicelluloses of well-rotted straw has been investigated with a view to obtaining information on possible resistant groupings.

2. Only very small quantities were obtained, and there was no indication of variation in availability or the accumulation of less available groupings. The complete availability of these substances to biological attack is emphasised.

3. The results indicate that the distribution and arrangement of the hemicelluloses in the cell-wall are such that microbial attack is not hindered by the presence of any resistant barrier.

4. A water-soluble polysaccharide, probably of microbial origin, was also prepared. It contained 33 % uronic acid anhydride, and 66 % hexosan, and gave evidence of the presence of glucose units.

The author is indebted to Sir J. Russell, Director of the Rothamsted Experimental Station, for placing at his disposal the facilities of the station; and to the Department of Scientific and Industrial Research for a Senior Research Award. His thanks are due to Mr E. H. Richards, Head of the Fermentation Department, for his assistance and advice.

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LXX. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET. IV. THE EFFECT OF PITRESSIN AND PITOCIN ON THE DISTRIBUTION OF FAT AND GLYCOGEN IN THE LIVER AND MUSCLES OF ALBINO RATS.

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(*Received March 14th, 1932.*)

In continuing the study of the distribution of glycogen and fat in the liver and muscles of animals under different nutritional conditions, described by us in previous papers [Hynd and Rotter, 1930; 1931, 1, 2], it was planned to investigate not only the effect of exogenous insulin but also the effects produced by the administration of those endocrine secretions, which are at present recognised as antagonistic to the action of the pancreatic hormone.

Though we have accumulated a considerable amount of data regarding the part played by the ductless glands concerned in the metabolism of carbohydrate and fat, the present paper is confined to a description of the results obtained with pitressin and pitocin, as other workers [Bischoff and Long, 1931] are evidently engaged in the same field. A full discussion of the significance of the results will be deferred until a later communication on the effect of pituitrin, and we hope, in subsequent papers, to deal with the effects of other ductless glands, namely the thyroid and the suprarenal.

It is now recognised that extracts of the posterior lobe of the pituitary body influence the metabolism of both fat and carbohydrate. The former was demonstrated experimentally for the first time by Coope and Chamberlain [1925], who found that the injection of pituitrin produced a fatty infiltration of the liver. This result, though denied by Van Dyke [1926], has been corroborated by other workers, and recently Raab [1930] has examined the effect on the blood-phosphatides of the two pituitrin constituents, "pitocin" and "pitressin," separated by Kamm *et al.* [1928]. Several years ago, Burn [1923] demonstrated that pituitrin had the property of checking the activity of insulin, and more recently this same worker [1929] arrived at the conclusion that the insulin-inhibiting effect of posterior lobe extract lay in the vasopressin ("pitressin"), and not in the oxytocin ("pitocin"). Magenta [1930], however, reached just the opposite view and reported that vasopressin and an anterior lobe extract did not modify the effect of insulin, while oxytocin was the active principle in pituitary-insulin antagonism.

In view of the contradictory reports in the literature, and the fact that the changes in glycogen and fat distribution have hitherto never been investigated simultaneously, it seemed desirable to undertake a further examination of the effects produced by the separated hormones of the posterior lobe of the pituitary.

Experimental.

The experiments here reported have been carried out on standard albino rats, 6–7 weeks old, the average body-weight being 80–100 g. The diets employed were (*a*) bread and milk for the carbohydrate-rich diet, and (*b*) cheese for the carbohydrate-free diet. The injections were made subcutaneously, the pituitary extracts used being "pitressin" and "pitocin" supplied by Parke, Davis and Co.

The effect of a single dose of the hormone, given at varying periods before death, was investigated. In this way it was possible to plot "time curves" of the changes in the blood-sugar, liver- and muscle-glycogen, and also liver-fat contents.

(A) *Effect of pitocin.*

The dose of pitocin employed was 20 units per 100 g. rat. Under this dosage, no definite effects on the general behaviour of the animals were noted, and the results of the chemical analyses, carried out on 12 rats, are given in Table I.

Table I. *Analyses of carbohydrate- and non-carbohydrate-fed rats after the subcutaneous injection of pitocin.*

Diet	Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
Carbohydrate-rich	0	0·15	0·55	3·5	3·7	120–140
	5	0·17	1·00	—	3·4	123
	7½	0·16	0·70	2·8	2·7	120
	7½	0·18	0·80	1·16	2·6	124
	15½	0·14	0·86	4·7	4·7	120
	15½	0·17	1·08	5·3	3·3	120
	16½	0·16	1·05	—	2·7	148
	18	0·16	1·00	4·5	2·0	124
Carbohydrate-free	0	0·17	0·63	1·9	5·0–7·0	100–128
	15½	0·16	0·94	1·6	4·9	121
	15½	0·17	1·26	1·6	5·7	—
	16½	0·15	0·73	1·0	9·7	102
	16½	0·15	1·00	1·4	8·5	116
	17	—	0·70	1·9	9·2	—

(B) *Effect of pitressin.*

After the injection of pitressin, many of the rats showed a distinct slowing of respirations. In some cases, slight sprawling of the limbs was noted, and nearly all the animals had a "blanched" appearance and felt cold when handled. These symptoms, however, lasted only for a short time after the injection, and probably correspond to the effect described by Lawrence and Hewlett [1925] as resulting after pituitrin in normal human subjects.

Table II. *Analyses of carbohydrate-fed rats after the subcutaneous injection of pitressin.*

Number of units	Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
2·5	0	0·15	0·55	3·5	3·7	120–140
	1	0·21	0·65	2·2	5·8	—
	1	—	0·82	2·2	4·6	92·5
	1¾	0·14	1·23	3·8	5·25	92
	2½	0·15	0·6	—	—	—
	2½	0·17	2·3	3·8	4·5	91·4
	5	0·19	0·96	1·15	6·0	88
	7	0·17	1·0	1·35	6·4	—
	12	0·23	0·65	6·75	6·1	99
	12	0·16	0·93	3·49	5·1	99
	17½	0·14	1·04	8·2	3·65	97·5
	24	—	1·4	5·5	4·8	104
	36	0·13	1·4	1·28	3·5	—
20	0	0·15	0·55	3·5	3·7	120–140
	¼	—	1·0	3·6	3·4	121
	½	0·21	0·66	3·5	3·5	148
	1	—	1·83	2·1	4·0	123
	1	0·24	—	2·1	6·15	—
	2½	0·16	0·77	2·2	5·2	99
	5	0·19	0·59	0·68	4·7	92
	7	0·16	1·0	2·0	4·2	82·5
	17	0·16	1·27	7·8	2·9	—
	17½	0·15	1·2	9·3	3·15	76
	17½	0·16	1·1	6·0	—	88
	24	0·17	0·99	4·8	2·55	136
	30	0·16	1·0	3·1	1·64	124
	36	0·17	1·52	3·2	3·0	—
40	0	0·15	0·55	3·5	3·7	120–140
	2½	0·14	0·81	1·0	5·0	86·5
	5	0·15	0·61	0·96	5·5	80
	5	0·15	0·62	1·2	5·1	97
	7	0·15	1·62	2·6	5·5	72
	12	0·16	—	4·4	4·3	112
	17½	0·16	1·24	8·7	3·7	87·7

Table III. *Analyses of non-carbohydrate-fed rats after the subcutaneous injection of pitressin.*

Number of units	Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
2·5	0	0·17	0·63	1·9	5·0–7·0	100–128
	1	0·13	0·93	1·18	5·0	139
	5	0·15	1·58	1·87	4·7	111·5
	12	0·15	1·3	2·6	6·8	84·3
	18	0·14	1·08	3·3	7·2	104·5
	22	0·16	1·05	2·7	13·1?	—
	24	0·15	0·71	4·1	6·9	95·4
	30	0·13	1·08	1·67	6·4	100
	42	0·15	0·95	1·1	—	88
20	17½	—	0·69	2·4	6·8	112
	17½	0·15	0·90	2·8	7·1	85·2
40	17	0·15	1·7	3·2	8·0	—

"Time curves" after the injection of 2·5, 20 and 40 units of pitressin per 100 g. rat were completed for carbohydrate-fed rats, and for non-carbohydrate-fed rats after 2·5 units of pitressin. Forty-six animals have been used in experiments with this hormone, and the results obtained are summarised in Tables II and III.

Results.

Inspection of the foregoing tables reveals the fact that pitocin is much less potent in its effect on either fat or carbohydrate metabolism than is pitressin.

With pitocin no alteration in blood-sugar was detected, as the values obtained were identical with those found in animals under similar dietetic conditions but receiving no injection. This is in agreement with the finding of Gavrila and Mihaileanu [1930] that pitocin was without influence on the blood-sugar level in the human subject, though it should be noted that Nitzescu and Benetato [1930] had previously reported that both pitocin and pitressin given intravenously brought about a marked and comparatively long-lasting hyperglycaemia in dogs and rabbits. Bacq and Dworkin [1930] obtained similar results with intravenously injected cats, namely a prompt rise in blood-sugar lasting for about 2 hours. As our first observation was made 5 hours after a subcutaneous injection of pitocin into a rat, the experimental conditions are not comparable. Consequently, the discrepancy between the results requires no further explanation.

Nevertheless, pitocin sometimes brings about an increase in muscle-glycogen. If this effect is achieved by a transference of glycogen from the liver to the muscles, it may furnish an explanation of the somewhat erratic results obtained for the liver-glycogen content. No definite changes in the liver-fat content were brought about by the subcutaneous injection of pitocin in carbohydrate-fed rats. The values obtained were irregular and may indicate a possible slight decrease in liver-fat content. With cheese-fed rats, the values obtained for liver-fat were somewhat higher than those for cheese-fed controls. However, as the iodine value of the fatty acids in the liver after pitocin remains normal, it is questionable whether the slight changes observed in either carbohydrate- or non-carbohydrate-fed rats, are of any significance.

In marked contrast with the indefinite character of the results obtained with pitocin, were the changes which ensued after the subcutaneous injection of pitressin. Slight hyperglycaemia was evident ½ hour after a dose of 20 units, but on the whole little variation in the blood-sugar level was observed. Nevertheless striking changes occurred in the liver-glycogen content, and also in the amount and character of the liver-fat.

In carbohydrate-fed rats, soon after the injection of pitressin, the liver-glycogen began to fall and the muscle-glycogen showed a tendency to rise. The liver-glycogen reached a minimum between the 5th and 7th hour, at which time the muscle-glycogen was definitely high. Thereafter, a rapid rise

in liver-glycogen occurred, a maximum of 6–8 % being attained about the 17th hour after the injection of the hormone. Subsequently there was a slow return to the normal level, which was reached by the 36th hour.

These changes are shown graphically in Fig. 1. The values for liver-glycogen after 2·5 units of pitressin are indicated by triangles, after 20 units by crosses and after 40 units by squares. The continuous curve is obtained by joining the average values found for the medium dose of 20 units. But it is evident that curves of similar type would result if either the average values for the small dose (2·5 units) or those for the large dose (40 units) were joined. Moreover, the maximum and minimum points of these curves would coincide with

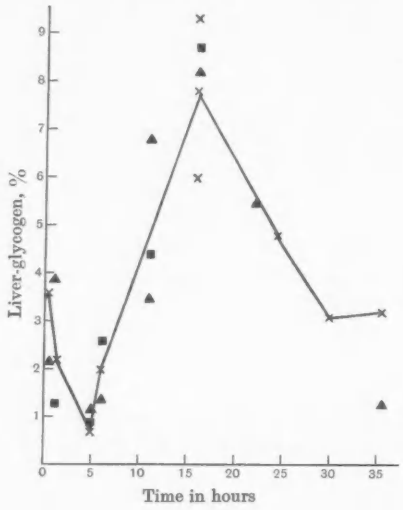

Fig. 1.

those of the curve drawn in Fig. 1, so that, as the same holds for the muscle-glycogen and also for the liver-fat, it appears that the effects produced by the various doses of the hormone are identical. No explanation of this somewhat remarkable result is attempted, but it may be correlated with the well-known fact that after repeated injections of the pressor principle of the posterior lobe of the pituitary, a tolerance to further administration tends to become established.

Recently, Bischoff and Long [1931] have reported that after pitressin "a possible but not clearly established increase in liver-glycogen takes place." This apparently conflicting result is easily explained, as these investigators made their glycogen determinations only at 5 hours after injecting the rabbit with pitressin, and it is clearly demonstrated in Fig. 1 that the liver-glycogen content varies with the interval elapsing after the injection of the hormone.

The results of the present investigation therefore confirm the increase in liver-glycogen noted by Nitzescu and Benetato [1928] to occur after pituitrin, but whereas this rise in liver-glycogen was accompanied by a fall in muscle-glycogen, reference to Table II shows that in our experiments with pitressin the muscle-glycogen tended to rise slowly to a final value approximately double the initial value (over 1 %). A transference of glycogen from the liver to the muscles might explain the fall in liver-glycogen observed during the first few hours and the coincident rise in muscle-glycogen, but such an explanation is quite inadequate to account for the high glycogen content of both liver and muscles at the 17th hour after the injection.

A study of the curves in Fig. 2 renders the simultaneous changes in liver- and muscle-glycogen, just referred to, as well as those in liver-fat content,

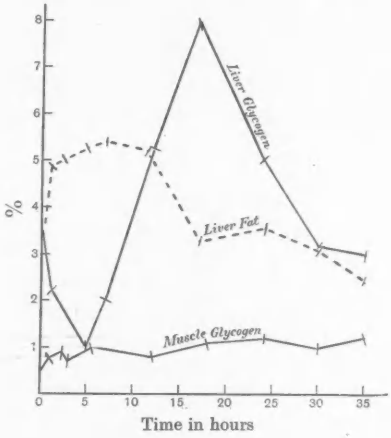

Fig. 2.

more easily appreciated. In carbohydrate-fed rats, pitressin produces an infiltration of fat, which is accompanied by a decrease in the iodine value of the fatty acids present in the liver. The rise in fat commences within an hour after the injection and may extend over a period of 12 hours—a maximum usually occurring about the 5th hour, at which time, it should be noted, the liver-glycogen content is at a minimum. Further, it is noteworthy that while the liver-glycogen passes from a minimum to a maximum between the 5th and 17th hours, during this period the liver-fat falls from a maximum to a more or less normal value.

With non-carbohydrate-fed rats, similar results were obtained. These are summarised in Table III and shown graphically in Fig. 3. It will be noticed that a possible slight increase occurred in muscle-glycogen, as after the first few hours it was maintained at a higher level than that found in cheese-fed

controls. Also, the initial fall in liver-glycogen was more rapid and the subsequent rise was less marked but more protracted than in the carbohydrate-fed animals. As in cheese-fed rats the liver-fat content was high (5–7 %) previous to the injection of pitressin, it was less certain whether the hormone caused a further infiltration of fat. But evidence that fatty infiltration occurred to some extent is furnished by the fact that the percentage of fatty acid between the 12th and 18th hours tended to be higher than those obtained earlier and later. Moreover, there was a decided fall in the iodine value which can only be explained by an alteration in the character of the liver-fat. Further, when the liver-glycogen is approaching its maximum value, the liver-fat content shows a tendency to decrease.

The results here recorded not only confirm the work of Coope and Chamberlain, but also point either to the pressor constituent of the posterior lobe of

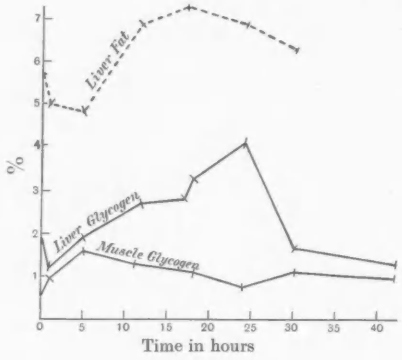

Fig. 3.

the pituitary, or to a hormone which accompanies it, being responsible for the infiltration of fat into the liver. Further, it is clearly demonstrated that that portion of the posterior pituitary extract in which the pressor constituent is concentrated also exercises a remarkable and somewhat unexpected effect on the glycogen reserves. Whether the hormone concerned with the effect on fat metabolism is identical with that affecting the metabolism of carbohydrate cannot be stated at present. Further experiments on this aspect of the problem are in progress and the results, as already indicated, will be communicated at an early date.

Summary.

No significant changes were observed to result from the subcutaneous injection of pitocin into rats on either a carbohydrate-rich or a carbohydrate-free diet.

Pitressin, on the other hand, produced striking changes in carbohydrate-fed rats.

As an early effect, there was observed slight hyperglycaemia accompanied by a fall in liver-glycogen and an increase in muscle-glycogen.

Simultaneously there occurred a marked infiltration of fat into the liver.

As the liver-fat content increased, the iodine value of the fat fell correspondingly.

The liver-fat reached a maximum at the 5th–7th hour, at which time the liver-glycogen was at a minimum, and the muscle-glycogen increasing.

The liver-glycogen reached a maximum about the 17th hour, at which time the liver-fat had returned to normal.

Similar but less striking results were obtained with non-carbohydrate-fed rats.

"Time curves" are given showing the simultaneous changes occurring in liver- and muscle-glycogen and liver-fat during a period of 36 hours after the injection.

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LXXI. A TITRIMETRIC METHOD FOR THE MICRO-DETERMINATION OF BARIUM.

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(*Received March 15th, 1932.*)

THE preparation and purification of substances of biological interest is in many cases facilitated through their isolation as barium salts. A knowledge of the percentage of barium present in these salts is often of importance in the study of their chemical nature. The determination of barium in small amounts has always, however, been attended with considerable difficulty. The well-known method of Pregl [1930] for sulphate is easily adapted to the determination of barium and gives excellent results, as also the newer Emich [1931] procedure for barium, using the filter-stick, which has proved so useful in micro-gravimetric analysis. Both these methods are thoroughly reliable, but require special apparatus, including a micro-balance, and a rather refined and specialised technique.

If the precipitate of barium sulphate be collected on a filter-paper, loss may occur through the finer particles passing through the pores of the paper. To overcome this difficulty McLaughlin [1931] proposed that the filtrate should be passed a second time through the paper to collect any particles which might have escaped during the first passage through the filter-paper. By this means he was able to show that his loss of barium sulphate did not amount to more than 0·3 % of the total, when dealing with precipitates of approximately 20 mg. in weight. Another troublesome source of error was the variable loss in weight due to reduction of some of the barium sulphate to barium sulphide in the presence of the hot reducing gases from the burner. These difficulties made it necessary to use electric heating in the final ignition of the precipitate.

An attempt was made to determine the conditions under which barium might be precipitated quantitatively as oxalate, in the hope of elaborating a procedure for barium similar to the Clark and Collip [1925] modification of Kramer and Tisdall's [1921] oxalate method for serum-calcium, in which the precipitate of calcium oxalate is collected and titrated in a small centrifuge-tube. Acidimetric titrations of the oxide formed from the oxalate on heating (similar to Fiske and Logan's [1931] method for calcium in urine), and of barium carbonate precipitated in a centrifuge-tube were also tried. Although some success was attained with these different procedures, and particularly with

barium oxalate, the results were often variable and inconsistent, and another indirect method involving the use of sulphuric acid and benzidine was eventually adopted as it was more satisfactory to apply and gave more reliable and consistent results.

A method for determining sulphate in urine in which benzidine hydrochloride was added to the diluted urine to precipitate the sulphate, and the benzidine sulphate filtered off and titrated with sodium hydroxide to a pink colour with phenolphthalein, was described by Rosenheim and Drummond [1914]. This method has been variously modified and adapted, particularly to total base determinations in serum, by several workers [Fiske, 1921, 1922; Stadie and Ross, 1925; Van Slyke, Hiller and Berthelsen, 1927], and has proved to be a useful and trustworthy procedure for the titrimetric estimation either of sulphate in solution or of sulphate in residues of total base.

The benzidine sulphate procedure may be adapted to the determination of small amounts of barium in solution. The barium is precipitated in a centrifuge-tube by an excess of standard sulphuric acid, and the excess determined by the benzidine procedure. The method has been in use in this laboratory for some time with good results. The results obtained have been repeatedly checked by comparison with gravimetric determinations (Emich) and have agreed sufficiently well to warrant the adoption of the method as a routine procedure.

Method.

Between 0·5 and 2 mg. of barium contained in 2 or 3 cc. of solution are measured into a conical 15 cc. centrifuge-tube, which is placed in a beaker of boiling water until hot. Exactly 4 cc. of standard 0·01 *N* H_2SO_4 are added to precipitate the barium and the contents of the tube thoroughly mixed. The tube is allowed to cool and 2 cc. of benzidine hydrochloride solution containing 4 g. benzidine and 50 cc. *N* HCl in 250 cc. are added to precipitate the excess of sulphuric acid. 3 cc. of 95 % acetone are now blown in from a fine-tipped pipette, so that the contents of the tube are well stirred up. After standing 10 minutes the sides of the tube are washed down with a little acetone, and the liquid centrifuged for 5 minutes. The supernatant liquid is siphoned off with the aid of a fine capillary tube bent up at the bottom, as in the Kramer and Tisdall [1921] method for calcium. It is best not to attempt to pour off the supernatant liquid at this stage, since some of the precipitate may be stirred up and lost.

The precipitate of barium sulphate and benzidine sulphate is washed in the centrifuge-tube by blowing in 3 cc. of the 95 % acetone, so as to impinge on the mat at the bottom of the tube, which is thus thoroughly stirred up. The sides of the tube are washed down with a further cc. of acetone, and the tube is again centrifuged for 5 minutes. The supernatant acetone layer may now be poured off carefully from the well packed precipitate and the tube inverted on a piece of clean filter-paper to drain for 2 minutes. The washing

is repeated and, after having centrifuged and drained the tube, 3 cc. of water are blown in so as to stir up the precipitate. The tube is placed in the beaker of hot water, a drop of phenolphthalein solution added and the benzidine sulphate titrated hot with 0·01 *N* NaOH to the first permanent faint pink colour. The barium sulphate does not appear to interfere in any way with the titration.

The calculation is as follows:

$$4{\cdot}00 - (\text{cc. } 0{\cdot}01\,N \text{ NaOH used}) = \text{cc. of } 0{\cdot}01\,N\ H_2SO_4$$

necessary to precipitate the barium. Each cc. of 0·01 *N* H_2SO_4 is equivalent to 0·687 mg. Ba, and therefore (4·00 – cc. 0·01 *N* NaOH) × 0·687 = mg. of barium in the sample taken.

Some examples of the results obtained with the method are given in the table.

Analysis of barium solutions by the benzidine method.

(1) Solution used	(2) cc. of 0·01 *N* NaOH to neutralise the benzidine sulphate	(3) cc. of 0·01 *N* H_2SO_4 pptd. by barium [4·00 – (2)]	(4) mg. of barium found [(3) × 0·687]	(5) mg. of barium present
2 cc. of a $BaCl_2$, $2H_2O$ solution (0·687 mg. Ba per cc.)	2·02	1·98	1·36	1·37
	2·09	1·91	1·31	
	2·11	1·89	1·30	
3 cc. of a $BaCl_2$, $2H_2O$ solution (0·687 mg. Ba per cc.)	1·10	2·90	1·99	2·06
	1·06	2·94	2·02	
1 cc. of a $BaCl_2$, $2H_2O$ solution (0·42 mg. Ba per cc.)	3·39	0·61	0·42	0·42
3 cc. of a $BaCl_2$, $2H_2O$ solution (0·42 mg. Ba per cc.)	2·19	1·81	1·24	1·26
3 cc. of a $BaCl_2$ (anhyd.) solution (0·65 mg. Ba per cc.)	1·03	2·97	2·04	1·95
	1·06	2·94	2·02	
				mg. of barium determined gravimetrically
Barium phenylphosphate solutions:				
3 cc. solution No. 1	2·29	1·71	1·17	1·16
	2·20	1·80	1·23	1·17
3 cc. solution No. 2	2·50	1·50	1·03	1·04
	2·48	1·52	1·04	
	2·43	1·57	1·08	
3 cc. solution No. 3	2·20	1·80	1·23	1·23
	2·24	1·76	1·21	
1 cc. solution No. 4	1·11	2·89	1·98	2·03
	1·16	2·84	1·95	2·02
3 cc. solution No. 5	2·16	1·84	1·26	1·24
3 cc. solution No. 6	2·18	1·82	1·25	1·27
	2·17	1·83	1·26	
				Calculated
Barium diphenylphosphate	2·42	1·58	1·09	1·07
Barium ethylphosphate 1 cc. approx. *M*/50 solution	0·23	3·77	2·59	2·58
	0·21	3·79	2·61	
Barium diethylphosphate 3 cc. *M*/100 solution	1·05	2·95	2·03	2·07
	1·03	2·97	2·04	
	1·05	2·95	2·03	
	0·95	3·05	2·09	

SUMMARY.

A titrimetric method for the micro-determination of barium is described, in which the barium is precipitated by an excess of standard sulphuric acid. The excess of sulphuric acid is determined by precipitation with benzidine and titration of the benzidine sulphate with sodium hydroxide.

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LXXII. WOOD CELLULOSE.

I. METHYLATION OF THE α-CELLULOSE.

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(*Received March 18th, 1932.*)

Recent contributions to the literature of cellulose chemistry reveal that the question of the identity of celluloses from different natural sources tends still to be investigated by methods which are in themselves essentially analytical and give only a superficial insight into the constitutional problems involved. Acylation, complete hydrolysis of the polysaccharide to glucose and solution of the cellulose in copper-ammonium solvents for the purpose of acquiring optical data are all accompanied by more or less extensive depolymerisation of the complex groupings of the constituent units and by complete disintegration of the natural organised structure. Deductions from the employment of such methods alone must be accepted with reserve, for it is well known that optical observations made on amorphous substances in colloidal, or semi-colloidal, solution are unreliable, and, by themselves, are not diagnostic. Many workers, however, from such evidence, have been led to believe in the existence of a close similarity between the celluloses of wood and of cotton [*vide e.g.* Heuser and Boedeker, 1921; Lenze, Pleus and Müller, 1921; Heuser and Aiyar, 1924; Hess *et al.*, 1925; Hess and Ludtke, 1927; Hägglund and Klingstedt, 1927, 1928; Arneman and Earl, 1929].

The successful subjection of cotton cellulose by Denham and Woodhouse [1913, 1914, 1917; Denham, 1921] in this Laboratory to methylation by the action of methyl sulphate and alkali, whereby the final product, containing its once free hydroxyl groups stabilised as ethers, was obtained in the fibrous condition, paved the way towards a more certain investigation of the constitution of the polysaccharides. Through the continuation of this work by Irvine and his school [1922, 1923, 1924, 1926] a probable structure for the basic unit of cotton cellulose has been suggested.

While Denham's discoveries have been widely applied by other workers in different branches of polysaccharide chemistry, it is noteworthy that only in two instances has the methylation process been applied to the cellulose of wood, and in neither case was more than a superficial examination made of the product. Urban [1926] methylated spruce wood to a maximal methoxyl content of 40 % but has reported no further investigation. Von Wacek [1928,

1930] subjected beech wood to methylation and found that etherification of the cellulosic portion of the wood ceased when a methoxyl content of 39–40 % was reached. After separation of the methylated cellulose from the other woody constituents, von Wacek found that the former exhibited partial solubility in water and in chloroform. From the hydrolysis products of the soluble portion some 2:3:6-trimethylglucose was isolated, indicating the presence within the wood cellulose molecule of the same grouping already known to compose the units of other polyglucosans.

The present work was undertaken in an endeavour to throw some light on the problem of the identity of the different wood celluloses, one with another, and each with cotton cellulose. The starting-materials were pure α-celluloses, specially prepared from the woods of *Quercus pedunculata*, *Q. sessiliflora*, *Fagus sylvatica*, and Sitka spruce, by Dr W. G. Campbell, of the Forest Products Research Laboratories. The author's thanks for this assistance are due to Dr Campbell; he is also grateful to Messrs Tullis, Russell, of Markinch, Fife, for a generous supply of "Thiriena pulp," a technical preparation of α-cellulose isolated by a particularly mild treatment of soft woods.

Methylation.

The wood celluloses were subjected to methylation by methyl sulphate and alkali, the reaction mixture being diluted with acetone. Etherification proceeded smoothly, and after a varying number of treatments, depending on the specimen of cellulose used, maximal methoxyl contents of 36·3 to 39·1 % were reached. A specimen of standard cotton cellulose treated under the same conditions attained a maximal methoxyl content of 44·3 % (calculated OMe for trimethylcellulose, 45·56 %). Here we have the first indication of difference between wood and cotton celluloses.

Further exhaustive treatment with methyl sulphate (10 to 18 methylations) or with Purdie's reagents failed to effect any increase in the methoxyl contents of any of the specimens of cellulose. The methylation experiments are detailed in Table I.

Table I.

Source of cellulose	No. of treatments	Maximal OMe %
Q. sessiliflora	10	36·3
Q. pedunculata	20	38·8
F. sylvatica	10	39·1
Thiriena pulp	18	39·0
Sitka spruce	12	37·3
Standard cotton	20	38·4
"	36	44·3

The specimen of cotton cellulose employed in the above experiments, although a technical "standard," was remarkable for its resistance to chemical action, which was not only displayed towards methylation, but also in other reactions such as acetylation.

Solubility of the methylated celluloses.

Urban [1926], Freudenberg and Braun [1928] and Haworth, Hirst and Thomas [1931] (who all employed Urban's modification of Denham's process) claim that their trimethylated cotton celluloses are soluble in chloroform and in other solvents. Haworth and his co-workers quote $[\alpha]_D - 10°$ for their material in chloroform solution. Urban's method, when tried by the author on three different cotton celluloses, failed to produce the results claimed for it; it may be that the celluloses in question were more resistant to treatment than were those available to the other workers, or that their products had undergone mechanical depolymerisation.

Exhaustive extraction with dry chloroform of the fully methylated celluloses of cotton and Thiriena pulp showed that both possessed partial solubility. That this was not due to differences in the degree of etherification existing between the soluble and insoluble portions was proved for both celluloses by the fact that the soluble portions, the insoluble residues and the intact methylated celluloses, in each case, possessed the same methoxyl content. The proportion of soluble material and the specific rotation of the latter differed markedly in the two cases under examination. Table II summarises

Table II.

Methylated cellulose	OMe %	Soluble material %	$[\alpha]_D$ in $CHCl_3$ ($c=1$)	Soluble fraction OMe %
Thiriena pulp	39·0	27·5	+ 2·5°	39·1
Cotton	44·3	37·8	−10·0°	43·9

this portion of the investigation. The insoluble residues appeared fibrous when examined microscopically; the soluble portions were deposited as clear colourless films on evaporation of chloroform solutions. No solvent could be found which could effect the complete solution of any of the methylated celluloses. Prolonged boiling with benzyl alcohol appeared to dissolve trimethylated cotton, but this solution was not examined.

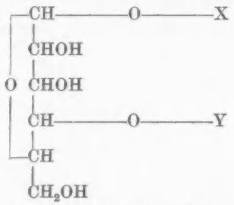
It appears that this partial solubility must be due to simple physical causes and not to any deep-seated structural differences. This is believed to be the first recorded evidence of such a non-homogeneity existing within the fibres of methylated celluloses, and of the fact that this condition exists in both wood and cotton celluloses.

Cleavage of the methylated celluloses.

On applying a modification of Irvine and Rose's [1906] method of simultaneous hydrolysis of the polysaccharide and condensation of the resulting cleavage-products with methyl alcohol containing hydrogen chloride, the unexpected and hitherto unrecorded fact emerged that these methylated wood celluloses each contained a fraction, incompletely methylated and totally resistant to hydrolysis. Moreover, after isolation, these "resistant portions," of about 28 % OMe content, could not be methylated above 32 % OMe.

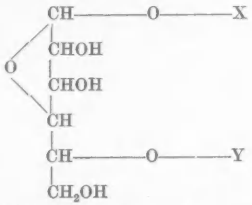
The remainder of the methylated cellulose, the non-resistant portion, gave rise, almost wholly, to 2:3:6-trimethyl-methylglucoside. Traces of dimethyl-methylglucosides, which appeared in some experiments, doubtless had their origin in incomplete methylation of physically masked hydroxyl groups. Tetramethyl-methylglucosides were in all cases definitely absent.

From the foregoing evidence it appears that the non-resistant fractions of the wood celluloses are composed of basic units similar to those comprising the bases of the other polyglucosans so far examined:



Amylene-oxide (stable) form

or



Butylene-oxide ("gamma") form

Which of the above structural formulae represents the true configuration of the unit we are at present unable to determine, since 2:3:6-trimethylglucose, the fission product of the methylated polysaccharide, can assume either the amylene- or the butylene-oxide ring-structure in its parent substance, while on isolation the stable amylene-oxide modification is always obtained.

Under the conditions of cleavage employed in the foregoing experiments, trimethylated cotton cellulose was completely broken down into the appropriate monosaccharide derivatives.

Table III records in detail the results of the cleavage experiments; the yields at all stages will be seen to be excellent, the care associated with quantitative analysis being bestowed on all the experiments.

Table III.

Source of cellulose	*Q. pedunculata*	*F. sylvatica*	Thiriena pulp	Sitka spruce
OMe % of methylate	38·8	39·1	39·0	37·3
"Resistant portion" %	40·1	23·9	22·5	19·1
"Resistant portion" OMe %	30·0	27·7	28·0	27·8
Cleavage products %	51·9	74·5	71·4	68·3

The cleavage products were in each case separated into trimethyl-methylglucoside and dimethyl-methylglucoside, and the pure 2:3:6-trimethylglucose isolated by hydrolysis of the glucoside. The yields of this sugar were of a consistently high order, as will be seen from Table IV.

Table IV.

Source of cellulose	*Q. pedunculata*	*F. sylvatica*	Thiriena pulp	Sitka spruce
Dimethyl-methylglucoside %	Nil	6·9	4·1	13·9
Trimethyl-methylglucoside %	100·0	93·0	94·9	86·1
2:3:6-Trimethylglucose, pure crystals %	95·8	90·0	93·1	97·2

Further communications regarding the investigation of the "resistant portions" and of depolymerised wood celluloses are in course of preparation.

Experimental.

Methylation.

Cellulose (1 part) was thoroughly soaked in 30 % sodium hydroxide solution (containing 2 parts NaOH). Acetone was then added, sufficient to form a layer about 1–2 cm. deep, on the surface of the alkaline suspension. The whole was heated to 45–50° on the water-bath and agitated by a mechanical stirrer, while equivalent portions of methyl sulphate (4 parts) and 30 % sodium hydroxide (2 parts NaOH) were added during the course of an hour. After the reagents had been added, stirring was continued for about 30 minutes, the reaction mixture heated to 90° on the water-bath and neutralised with acetic acid. The solid product was separated by filtration on a sintered glass funnel heated by means of an immersed steam-coil. Hot filtration is essential during the early stages of methylation owing to gelatinisation of the product at temperatures below about 70°, and its partial solubility in the cold alkaline liquors.

The product of the above treatment was remethylated if desired. Specimens for analysis were thoroughly washed with boiling water, dried in the steam-oven and finally extracted three times with changes of boiling acetone. By this means a yellow gum, of low methoxyl content, was removed, leaving the methylated cellulose as a white fibrous material. This yellow gum probably originated in the action of the alkali on the acetone used in the methylation process. It was found essential to ensure that the purified specimens contained no occluded organic solvents. A specimen of methylated cotton cellulose, after washing with alcohol and ether and drying for an hour at 100°/10 mm., showed an apparent methoxyl content of 45·6 %. After further heating for an hour at 120°/0·1 mm. the value of 44·3 % was obtained, which was in accord with the methoxyl content calculated from the cleavage products.

Cleavage of the methylated wood celluloses.

The following method, after many trials of variation in conditions, was found to effect a clean-cut separation into "resistant" and hydrolysed portions, with absence of decomposition. Methylated cellulose (1 part) was heated in a sealed tube at 80° for 400 hours with methyl alcohol (10 parts) containing 1 % of dry hydrogen chloride. The insoluble "resistant portion" was separated by filtration on a tared glass filter and washed with methyl alcohol, it was then dried to constant weight and weighed.

The methyl alcohol solution of the cleavage products was neutralised with silver carbonate, filtered and evaporated to half bulk. An equal volume of ether was added and the whole boiled with a little norite to remove colloidal silver. The solution was filtered into a tared flask and evaporated to dryness

under reduced pressure, when the cleavage products were obtained as a clear, usually colourless syrup.

The cleavage products were then separated into their constituents by first dissolving the syrup in the minimum of cold water. Twenty extractions with chloroform then sufficed to separate quantitatively all the trimethyl-methylglucoside (and any pentamethylglucose, if present).

Isolation of the dimethyl-methylglucosides from the aqueous solution was effected by a new method. The addition of solid potassium carbonate to the solution of the glucoside until its specific gravity is just less than that of water-saturated chloroform renders it possible to effect complete separation of the glucoside by 15 to 20 extractions with chloroform.

The chloroform solutions of the glucosides were dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. Further purification by distillation in high vacua was resorted to if found necessary.

Aqueous acid hydrolysis of the trimethyl-methylglucoside showed that the sugar was methylated in positions 2, 3 and 6. Chloroform extraction of the aqueous solutions of the sugar failed to reveal any trace of tetramethyl-sugars, even when carried out on an extensive scale. The 2:3:6-trimethyl-glucose was always obtained in a very high state of purity, as indicated by its constants and the ease with which it crystallised.

Typical cleavage experiments are detailed below.

(*a*) *Methylated cellulose from* Q. pedunculata (OMe, 38·8 %).

5·389 g. material yielded resistant portion: 2·207 g. (OMe, 30·8 %); cleavage products: 3·260 g. (n_D^{17} 1·4600).

3·260 g. of above cleavage products contained 3·260 g. material extractable from water with chloroform, therefore no dimethylglucoside was present. 1·450 g. of the trimethyl-methylglucoside on aqueous acid hydrolysis yielded 1·214 g. (95·8 %) of trimethylglucose, M.P., after one recrystallisation from dry ether, 117–120°.

(*b*) *Methylated cellulose from* F. sylvatica (OMe, 39·1 %).

14·074 g. material yielded resistant portion: 3·376 g. (OMe, 27·7 %); cleavage products: 11·630 g.

The cleavage products were separated into 10·497 g. (90·3 %) pure trimethyl-methylglucoside, 0·236 g. (1·7 %) of unhydrolysed non-resistant material, and 0·785 g. (5·6 %) dimethyl-methylglucoside. Aqueous acid hydrolysis of 10·094 g. of trimethyl-methylglucoside yielded 8·549 g. (90·0 %) of crystalline 2:3:6-trimethylglucose, M.P., after one recrystallisation, 115°.

(*c*) *Methylated cellulose from Thiriena pulp* (OMe, 39·0 %).

4·637 g. material yielded resistant portion: 1·041 g. (OMe, 28·0 %); cleavage products: 3·823 g.

3·711 g. of cleavage products were separated into 3·423 g. (92·2 %) trimethyl-methylglucoside, and 0·188 g. (4·95 %) dimethyl-methylglucoside,

29·471 g. of trimethyl-methylglucoside yielded 25·810 g. (93·1 %) crystalline 2:3:6-trimethylglucose, M.P., after one recrystallisation, 113–117°.

(*d*) *Methylated cellulose from Sitka spruce* (OMe, 37·3 %).

12·430 g. material yielded resistant portion: 3·618 g. (OMe, 27·8 %); cleavage products: 9·839 g.

9·300 g. of cleavage products were separated into 7·907 g. (85·0 %) trimethyl-methylglucoside and 1·191 g. (12·8 %) dimethyl-methylglucoside. 7·308 g. of trimethyl-methylglucoside yielded 6·682 g. (97·2 %) crystalline 2:3:6-trimethylglucose, M.P., after one crystallisation, 116–118°.

SUMMARY.

1. The process of methylation has been applied to five specimens of wood α-cellulose. The maximal methoxyl contents attainable under conditions which will effect the complete etherification of standard cotton cellulose lie between 36·3 and 39·1 % according to the source of the material.

2. Cleavage of the methylated wood celluloses revealed the hitherto unsuspected presence within them of "resistant portions," which have not been detected in those celluloses previously investigated.

3. The non-resistant portions are constructed wholly of glucose residues, having free hydroxyl groups in positions 2, 3 and 6.

4. Both cotton and wood celluloses, when fully methylated, exhibit partial solubility in chloroform, which is apparently due to the existence within the methylated cellulose fibres of different degrees of polymerisation of similar basic units. The soluble fractions display, in chloroform, the following specific rotations—cotton, −10°, Thiriena pulp (wood), +2·5°.

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LXXIII. WOOD CELLULOSE.
II. THE DEPOLYMERISED CELLULOSES OF WOOD AND COTTON.

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(*Received April 1st, 1932.*)

In a previous communication [1932] on the behaviour of wood α-celluloses towards methylation and the subsequent cleavage of the methylates, it was demonstrated that these α-celluloses could not be completely etherified with respect to the three hydroxyl groups known to be present in the C_6 unit. Treatment with methyl sulphate and alkali, by a method adequate to accomplish complete methylation of cotton cellulose, and of starch [Bell, 1931] (a notoriously resistant polysaccharide) brought about etherification to the extent of only 36·3–39·1 % of methoxyl. Cleavage of the methylated wood celluloses, under conditions promoting complete fission of trimethylated cotton cellulose, resulted in the dissection of the materials into two parts.

(*a*) A fully methylated fraction, reacting with the cleavage reagents and manifesting itself as a virtually homogeneous cleavage product consisting of 2:3:6-trimethyl-methylglucoside.

(*b*) A "resistant portion," methoxyl content 27–30 %, insoluble in all solvents, and completely resistant to cleavage by any known means. By exhaustive methylation the methoxyl content could be raised to 30–32 % but no further.

Since these "resistant portions" appeared to have a hitherto unsuspected existence in all the four wood celluloses examined, an investigation of their properties and, if possible, an elucidation of their inert behaviour, appeared to be of importance. The obvious explanation of the non-reactive behaviour is that of a high polymerisation within these fractions, a condition not obtaining in either cotton or esparto cellulose.

Were high polymerisation the reason, and could depolymerisation of this ultra-complex fraction be effected, then resistance should disappear.

Acetylation by Barnett's method [1921] appeared, from experiments on starch carried out in this laboratory by Irvine and Rutherford [1932], to effect considerable depolymerisation of intact polysaccharides. It was therefore considered that the application of this process to wood celluloses might afford a possible means of determining whether or not the behaviour of the "resistant

portions" was a result of high polymerisation. Subsequent methylation of the acetate, followed by cleavage of the resulting methylate, would provide definite information as to whether or not resistance survived acetylation and depolymerisation. A retention of non-reactivity would be a strong reason for assuming that mere molecular complexity was not the basal cause of the properties of the "resistant portions."

Acetylation.

The action of Barnett's reagents on the wood celluloses, under carefully standardised conditions, resulted in the production of triacetates in almost theoretical yields. The specific rotations of the acetates, in chloroform solution, showed various values, as will be seen from Table I. The alkali-extracted

Table I.

Source of cellulose	Yield of triacetate %	$[\alpha]_D$ in $CHCl_3$
F. sylvatica	97·3	−19·6°
Thiriena pulp	98·4	−18·4°
Q. sessiliflora	94·5	−11·9°
Sitka spruce	96·7	−10·4°
Q. pedunculata	97·2	0·0°
Controls:		
Standard cotton	97·6	−22·7°
,, (alkali-extracted)	96·8	−23·0°

cotton was prepared by extraction with 12 % NaOH at 90° for 9 hours. The specific rotation of the acetate shows that alkali-extraction does not account for the low values found for the wood acetates.

The wood acetates were all non-reducing, contained no pentose residues, and were soluble in highly halogenated paraffins, but not in alcohol or acetone. Acetyl estimations, in general, gave slightly high values for triacetates, but no significance can be attached to this fact bearing in mind the nature of the processes involved in this determination.

The simultaneous de-acetylation and methylation of wood-cellulose acetate ($[\alpha]_D - 18·4°$).

Employing the procedure of Irvine and Robertson [1926], methylation of the depolymerised Thiriena pulp cellulose was found to take place with greater ease than that of the corresponding intact cellulose. Ten treatments effected the introduction of methyl groups amounting to 39·2 % OMe, and exhaustive treatment resulted in no increase. Yields amounted to 91·7–94·0 % of that theoretically possible. The methylated depolymerised cellulose was a white powder, soluble in chloroform, benzene, pyridine, alcohol and glacial acetic acid. It was insoluble in acetone. In most of these solvents the solutions were clear, easily filterable and of inconsiderable viscosity. In 5 % solution in chloroform the methylate had a specific rotation of +2·3°, a value in close

agreement with that of the chloroform-soluble fraction of methylated intact Thiriena pulp [Bell, 1932].

From this last observation, if the doubtful criterion of optical data be adopted, the possibility of depolymerisation of the insoluble fraction of the cellulose to the same level of complexity as the chloroform-soluble one must not be overlooked.

Comparative experiments on depolymerised cotton cellulose.

Cotton cellulose triacetate, $[\alpha]_D - 22{\cdot}7°$, when treated in the same fashion as the Thiriena pulp acetate, attained a methoxyl content of 43·7 % after 14 methylations. No increase in the methoxyl content could be effected, either by the use of methyl sulphate and alkali on a pyridine solution of the methylate, or by the action of Purdie's reagents on the material dissolved in dry chloroform. It is noteworthy that Purdie's reagents effected no apparent change in the methylated depolymerised cotton, while considerable alteration in the condition of methylated starch takes place under similar circumstances, as has been shown by Irvine and Macdonald [1926] in this laboratory.

Methylated depolymerised cotton was a white powder, possessing the same solubilities as methylated depolymerised wood cellulose. In chloroform solution it showed $[\alpha]_D - 1{\cdot}7°$; after several treatments with Purdie's reagents the value was found to be virtually the same, namely $-1{\cdot}2°$. In this case, it will be observed, there is no agreement between the rotations of the depolymerised methylate and the soluble fraction of intact trimethylated cotton [Bell, 1932].

Eight methylations of the cotton acetate introduced 38·6 % of methoxyl; specimens of this material were employed in comparative cleavage experiments with the methylated derivative of Thiriena pulp.

Acetylation of the "fully-methylated" depolymerised cotton (OMe, 43·7 %) showed that free hydroxyl groups were still present, although they could not be etherified. For the acetyl derivative, an acetyl content of 2·90 % was found; theory 2·63 %.

The investigation of the celluloses of wood and cotton, both intact and depolymerised, is summarised in Table II.

Table II.

Cellulose	Cotton		Wood	
	Intact	Depolym.	Intact	Depolym.
$[\alpha_D]$ of triacetate	−22·7°	—	−18·4°	—
Treatments required for complete methylation	36	14	18	10
OMe of methylate	44·3 %	43·7 %	39·0 %	39·2 %
OMe of methylate (soluble fraction of intact cellulose)	43·9 %		39·1 %	
$[\alpha]_D$ of methylate (soluble material)	−10·5°	−1·2°	+2·5°	+2·3°

The properties of the trimethylated depolymerised cotton are in almost exact agreement with the values given by Hess *et al.* (1923] for his trimethylcellulose "A" in the crude state. In no way did the material resemble the "trimethylcellulose" which Haworth, Hirst and Thomas [1931] claim to have obtained with exceptional facility from an acetone-soluble cellulose acetate. (Attempts to repeat the experiments of these workers, it may be stated, were not successful.) The three substances under consideration are compared in Table III.

Table III.

	Hess	Bell[1]	Haworth
$[\alpha]_D$ of initial acetate	−16·7°	−22·7°	—
Trimethylcellulose, OMe	42 %	43·7 %	45·6 %
" $[\alpha]_D$ in $CHCl_3$	0·0°	− 1·2°	−10°
" M.P.	230–245°	233–240°	215–216°*

* Material of OMe 41–42 % had M.P. 242–243°.

Subsequent to their original experiments, Hess and Schultze purified the cellulose acetate "A," whereby the rotation in chloroform increased to −19·5° [1927]; a genuine "trimethylcellulose A" (OMe, 45 %) was also obtained from the crude product [Hess and Weltzien, 1925].

Relation between the specific rotation of the wood cellulose acetates and the proportion of the "resistant portion" in the material.

What interpretation, if any, can be placed on the magnitude of rotation of a cellulose acetate? A host of reducing and non-reducing derivatives of cellulose of widely different degrees of apparent complexity display closely similar optical activity. Ost and Kayama [1912] from alkali-treated cellulose have obtained acetone-soluble triacetates showing $[\alpha]_D$ −17° to −22°. The "hexacetyl anhydrobiose" of Hess [1921], $[\alpha]_D$ −17·8°, his "triacetyl cellulose 'A,'" and the wood cellulose triacetates described above are examples which appear only to confuse attempted explanation.

A consideration of the wood cellulose acetates reveals that an almost quantitative relationship exists between their specific rotations and the proportion of resistant material obtained in the cleavage of the corresponding methylates [Bell, 1932] (see Table IV). While the relationship is not uniformly

Table IV.

Source of α-cellulose	$[\alpha]_D$ of acetate in $CHCl_3$	% "resistant portion"
F. sylvatica	−19·6°	24
Thiriena pulp	−18·4°	23
Sitka spruce	−10·4°	30
Q. pedunculata	0·0°	40

constant, it is apparent that an increase of 1 % in the content of "resistant portion" results in an increase in dextro-rotation of approximately 1°. While

[1] Dr N. K. Adam has informed the author, after this paper had been sent to the printer, that this depolymerised product gives surface-film results identical with those given by the trimethylcellulose of Hess.

this observation, together with a consideration of the results of the methylation experiments, suggests profound differences between the nature of the "resistant portions" and that of the remainder of the wood cellulose, the investigation of the "resistant portions" has by no means been confined to such empirical observations. This side of the problem will be dealt with in a forthcoming communication.

Cleavage of the methylated depolymerised cellulose.

The derivative of Thiriena pulp was subjected to the action of methyl alcohol containing hydrogen chloride; at the same time fully methylated (OMe, 43·7 %) and partially methylated (OMe, 38·6 %) depolymerised cotton celluloses were treated under the same conditions. Employing the technique described previously [1932] for the cleavage of the methylated intact celluloses, the following results were obtained.

Table V.

Methylated cellulose (depolymerised)	Thiriena pulp (39·2 % OMe)	Cotton (43·7 % OMe)	Cotton (38·6 % OMe)
Duration of treatment	400 hrs.	400 hrs.	400 hrs.
Cleavage products isolated	81·9 %	93·0 %	95·0 %
2:3:6-Trimethyl-methylglucoside	92·3 %	83·5 %	—
Dimethyl-methylglucoside*	7·2 % (*a*)	13·8 % (*b*)	—
Resistant material found	14·0 %	Nil	Nil
Methoxyl content of resistant material	29·9 %	—	—

* These materials had OMe (*a*) 36·5 %; (*b*) 38·4 % values indicating contamination with lower homologues.

Both the cotton derivatives were completely broken down; on the other hand the wood methylate, initially soluble in the cleavage reagent, had become split into soluble cleavage products and an insoluble resistant residue. The proportion of the latter was decreased compared with the amount of resistant material obtained from the methylated intact Thiriena pulp, 14 % in place of 22·5 %, but the amount of dimethyl-methylglucoside among the cleavage products was relatively increased.

A comparison of the cleavage experiments on the methylates of intact and depolymerised Thiriena pulp is of interest (see Table VI).

Table VI.

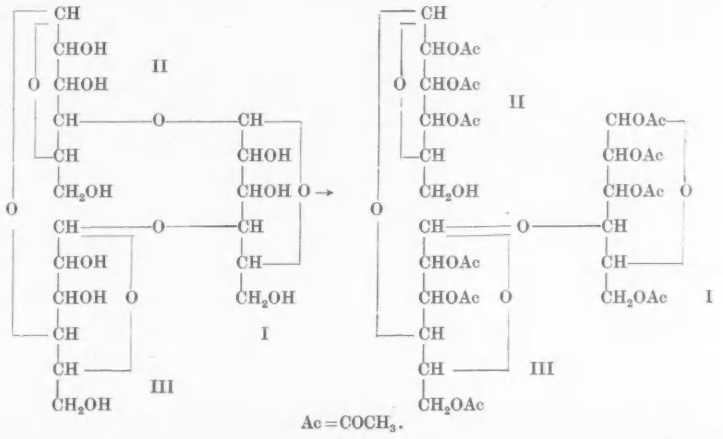
Methylate of Thiriena pulp	Intact %	Depolymerised %
Methoxyl content	39·0	39·2
"Resistant portion"	22·5	14·0
Methoxyl content of "resistant portion"	28·0	29·9
Cleavage products	71·4	81·9
Trimethyl-methylglucoside	94·9	92·3
Dimethyl-methylglucoside	4·1	7·2
Ratio $\frac{\text{Trimethyl-methylglucoside}}{\text{Dimethyl-methylglucoside}}$	23·1	12·8

From the above, and preceding results, it will be seen that the inert properties of the "resistant portion" have been proved to survive acetylation,

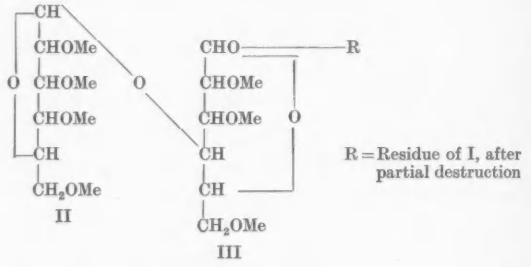
disintegration of the cellulose fibre and extensive depolymerisation of the polysaccharide to a much less complex state of molecular aggregation. It must be noted, however, that the "resistant portion," although still incompletely etherifiable, had become so depolymerised as to be no longer completely resistant to cleavage. The evidence in favour of this resistance being due to conditions more fundamental than mere high polymerisation seems definitely convincing.

The presence of tetramethylglucose among the cleavage products of methylated celluloses.

Haworth [1932] reports the detection of tetramethylglucose from the cleavage products of a "trimethylcellulose" obtained by methylating acetone-soluble cellulose acetates. This is claimed to be a proof that polysaccharides are constructed on the basis of long, open chains of glucose residues, and it is further proposed that his method affords a clue to the length of the said chains. How far this method may be applied to starch is dealt with by Irvine in a reply to Haworth's suggestions [1932]. Haworth's views do not lend themselves to acceptance without criticism. The extreme ease with which he introduces methyl groups into celluloses, after subjecting them to preliminary treatment, suggests at once that extensive depolymerisation, possibly not unaccompanied by degradation also, has occurred in his materials. Moreover, the production of an acetone-soluble acetate of cellulose requires an intensified treatment, and it is not unreasonable to suspect the possibility of degradation of a closed system occurring, as indicated in the following scheme. (A ring-structure of three anhydroglucose units is employed for convenience only, no suggestion is thereby made of the number of C_6 chains composing the cellulose unit.) The linkage between chains I and II has been acetolysed with the introduction



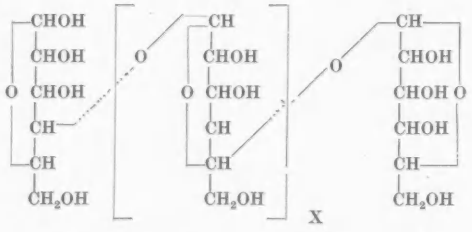
of acetyl groups in position 4 of chain II and position 1 of chain I. If the same conditions for destruction of reducing groups by alkali hold for polysaccharides as for simple sugars, we should expect, during methylation, that destruction of the major part of chain I would occur, with the result that, after methylation, the following condition would obtain:



On cleavage of the methylate, we should obtain, for every complex unit of n C_6 chains which has undergone degradation at one linkage,

(n − 2) molecules of 2:3:6-trimethylglucose,
1 molecule of tetramethylglucose,
i.e. (n − 1) C_6 units recovered.

The occurrence of tetramethylglucose as a cleavage product from an initially closed ring, is therefore rendered possible, and it is not necessary to postulate a chain structure, such as the following, to account for its presence:



Careful examination of the cleavage products of fully-methylated intact cotton cellulose failed to detect any trace of tetramethylglucose. From 20 g. of crude trimethyl-methylglucoside, by the method described in the Experimental Section, complete absence of even 0·0005 g. (0·0025 %) of tetramethylglucose was proved.

By similar treatment, methylated intact wood cellulose (Thiriena pulp) was shown to yield no tetramethylglucose. After depolymerisation, however, this wood cellulose was found to yield about 0·5 % of the weight of the methylate, as pure, crystalline 2:3:4:6-tetramethylglucose. This is strong evidence of degradation having taken place during acetylation.

Examination of the cleavage products of depolymerised cotton, methylated to the extent of (*a*) 43·7 % OMe, *i.e.* fully-methylated; and (*b*) 38·6 % OMe, *i.e.* approximating to the OMe content of a fully-methylated wood cellulose, yielded a result at present inexplicable. No trace of tetramethylglucose was found in the case of (*a*), but cleavage of (*b*) gave rise to 0·64 % of 2:3:4:6-tetramethylglucose. In the extremely empirical state of our present knowledge of polysaccharide chemistry, it seems impossible to proffer any feasible explanation of this phenomenon.

Experimental.

Acetylation.

One part of cellulose was suspended in glacial acetic acid (10 parts) and chlorine bubbled through the mixture (for 10 secs. per 10 cc.). Acetic anhydride (10 parts) was then added and sulphur dioxide passed through the whole for twice as long as the time taken for the chlorine. The reaction mixture was heated to 75° on the water-bath and stirred until the solid matter dissolved, forming an almost clear, viscous solution. This was poured into a large excess of water containing sodium acetate, and the bulky solid which separated was collected, washed acid-free and dried at 100°. Purification was effected by dissolving the crude product in chloroform, drying the solution over anhydrous sodium sulphate, filtering through a sintered glass funnel and evaporating under diminished pressure. The triacetates were deposited as tough colourless films, soluble in chloroform, but insoluble in alcohol and acetone. The chloroform solutions, although clear, were too viscous to filter through paper. Observations of optical activity were made on 1 % solutions. Stronger solutions possessed too great a power of light-dispersion to permit of accurate measurement.

The experimental results are given in Table VII.

Table VII.

Source of cellulose	Weight of starting material g.	Yield	Acetyl content %	Specific rotation in $CHCl_3$
Standard cotton	—	(97·6 %)	45·0	−22·7°
Alkali-extracted cotton	—	(96·8 %)	45·2	−23·9°
Fagus sylvatica	1·013	1·784 g. (97·3 %)	45·5	−19·6°
Thiriena pulp	4·00	7·00 g. (98·4 %)	47·4	−18·4°
Quercus sessiliflora	4·5	7·8 g. (94·5 %)	46·9	−11·9°
Sitka spruce	1·048	1·802 g. (96·7 %)	46·8	−10·4°
Quercus pedunculata	2·2	3·85 g. (97·2 %)	47·4	Inactive

Theory for $C_6H_7O_5(COCH_3)_3$; $COCH_3$ 44·7 %.

Simultaneous de-acetylation and methylation of cellulose triacetates.

The process was exactly similar to that described previously for the intact celluloses [Bell, 1932], with the exception that the acetates, prior to treatment with the methylating reagents, were stirred for 3 hours with acetone and 30 % sodium hydroxide solution in amount slightly in excess of that required to effect de-acetylation.

(*a*) *Wood triacetate* (*Thiriena pulp*). 35 g. of material after 10 treatments yielded 22 g. of a fine white powder; yield 91·7 % of theory, methoxyl content 39·2 %. Six further treatments failed to raise the methoxyl content. The material was dissolved in chloroform and shaken for 10 minutes with a little norite charcoal. After filtration through a bed of solid barium carbonate a perfectly clear, pale yellow solution was obtained which, on evaporation under diminished pressure, yielded a tough, very pale yellow film. The specific rotation of this material (5 % solution in chloroform) was $+2{\cdot}3°$.

(*b*) *Cotton cellulose triacetate.* 65 g. of the triacetate were treated as in (*a*). After 9 methylations 38 g. (85·5 % yield) of product with a methoxyl content of 38·6 % were obtained.

19 g. of this substance were subjected to 5 more methylations, the final product amounted to 18 g. and showed a methoxyl content of 43·7 %.

Further treatment with methyl sulphate and alkali failed to produce any increase in the methoxyl content; treatment of a pyridine solution of the material was likewise without avail. Purification was effected as in (*a*), but as the solution could not be freed from a trace of cloudiness, the specific rotation could not be determined with accuracy. A 3 % solution gave $[\alpha]_D - 1{\cdot}7°$ ($\pm 0{\cdot}5$) and the substance melted at 233–240°.

The action of Purdie's reagents on the above product.

3·475 g. of the methylated depolymerised cellulose were dissolved in the minimum of dry chloroform and treated with 15 g. methyl iodide and 15 g. silver oxide. The product was isolated in the usual way and treated twice as before. The final product gave a perfectly clear solution in chloroform; $[\alpha]_D - 1{\cdot}2°$ in 5 % solution. The methoxyl content was 43·9 % and the M.P. 233–240°.

Cleavage of the methylated depolymerised celluloses.

The cleavage was effected under the same experimental conditions as described for the treatment of the methylated intact celluloses [Bell, 1932].

(*a*) *Material from Thiriena pulp.* 5 g. were treated at 80° for 400 hours; a solid residue was found at the end of this time amounting to 0·701 g. (14·0 %) and having a methoxyl content of 29·9 %. The cleavage products weighed 4·351 g. These were separated into 4·029 g. of trimethyl-methyl-glucoside, $n_D^{17°}$ 1·4587, and 0·317 g. (7·2 %) of residual material, $n_D^{18°}$ 1·4792, of methoxyl content 36·5 %.

3·246 g. of the above trimethyl-methylglucoside yielded 2·585 g. (84·7 %) of pure crystalline 2:3:6-trimethylglucose, M.P. 119–120°.

(*b*) *Material from cotton* (*OMe, 43·7 %*). 2·443 g. were treated as in (*a*). Complete hydrolysis took place, the cleavage products amounting to 2·658 g. (93·0 %). Separation was effected into 2·273 g. trimethyl-methylglucoside (corresponding to 85·6 % of the methylated cellulose; theory, calculated from the methoxyl content, 88 %), and 0·368 g. of residual material (13·3 % of the methylated cellulose).

Examination of the cleavage products for tetramethylglucose.

This was carried out by first subjecting the cleavage products to the chloroform-water separation. The chloroform extract was distilled in a high vacuum (0·05 mm.) and the first runnings of the distillate, in which the pentamethylglucose becomes concentrated, collected separately and submitted to aqueous acid hydrolysis. The solution of sugar was extracted 20 times with chloroform and the extract worked up in the usual way. The extracted substance, if any, was weighed on the analytical balance, whereby 0·0005 g. of material could be definitely detected.

(*a*) *Material from Thiriena pulp.* 10·893 g. of syrup yielded 0·081 g. of colourless syrup which set to a hard mass of crystals after nucleating with 2:3:4:6-tetramethylglucose. After recrystallising once from light petroleum the substance melted at 87°. A mixed M.P. with 2:3:4:6-tetramethylglucose showed no depression. The amount of tetramethylglucose isolated corresponded to 0·5 % of the original methylate.

(*b*) *Material from cotton* (*partially methylated*). From 6·730 g. of depolymerised methylated cotton, 0·048 g. of crystalline tetramethylglucose were isolated, M.P. 88°; a mixed M.P. with 2:3:4:6-tetramethylglucose showed no depression.

(*c*) *Material from cotton* (*fully methylated*). No trace of tetramethylglucose could be detected, even after most careful examination.

Summary.

1. Acetylation of different wood α-celluloses, under standard conditions, produced triacetates of specific rotations varying from −19° to inactivity.

2. Simultaneous de-acetylation and methylation of the acetate led to the formation of fully-methylated depolymerised cellulose (OMe, 39·2 %), completely soluble in most organic solvents, and having, in chloroform solution, $[\alpha]_D = -2{\cdot}3°$. The empirical composition is the same as that of the corresponding intact cellulose.

3. Cleavage of the above methylate yielded, as in the case of the intact methylate, 2:3:6-trimethyl-methylglucoside, and a "resistant portion," with 29·9 % OMe.

4. No tetramethylglucose was detected in the cleavage products of fully-

methylated intact wood cellulose, but examination of the cleavage products of the same cellulose, after depolymerisation and methylation, proved the presence of a small amount (0·5 % of the starting material) of 2:3:4:6-tetramethylglucose.

5. Control experiments on standard cotton cellulose showed that acetylation also effects depolymerisation, the trimethylated depolymerised cellulose being soluble in most solvents and having in chloroform solution $[\alpha]_D = -1{\cdot}2°$. Cleavage of this material was complete, and no tetramethylglucose was found.

6. Cleavage of depolymerised cotton methylated to the extent of 38·6 % OMe was complete, no resistance being encountered. In this case, however, 0·64 % of the starting material was isolated as 2:3:4:6-tetramethylglucose.

7. A definite relationship has been indicated between the optical activity in chloroform solution of the wood cellulose acetates and the proportion of "resistant material" isolated from the corresponding methylated intact celluloses.

8. It has now been shown that marked differences are to be found between cotton and wood celluloses; further, the wood celluloses do not display uniformity as individuals, but only as a class, possessing in varying proportions, a "resistant portion." The "resistant portion" has been shown to owe its inert properties to more fundamental reasons than degree of polymerisation.

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LXXIV. WOOD CELLULOSE.
III. THE "RESISTANT PORTIONS."

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(*Received April 30th, 1932.*)

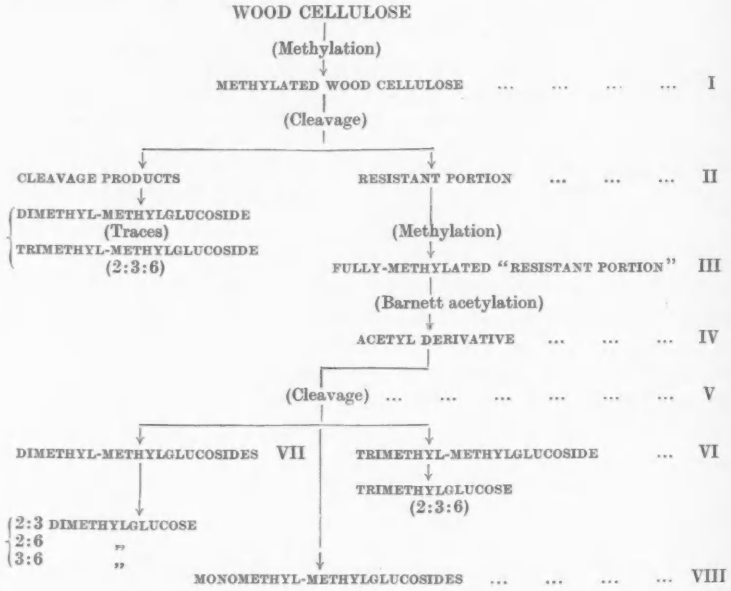
THE application by the author [1932, 1] of the methylation process to α-celluloses of wood has revealed a definite difference between wood cellulose and that from cotton and esparto, the only other varieties similarly investigated. Etherification of the wood celluloses could not be carried beyond a methoxyl content of 37–39 % despite the fact that, under identical conditions, a very resistant specimen of cotton could be methylated almost to the theoretical maximum. Cleavage of the wood methylates yielded a fully-methylated portion which manifested itself as 2:3:6-trimethyl-methylglucoside. The remainder of the starting material was apparently unaffected, appeared as an insoluble amorphous substance of methoxyl content 27–30 %, and proved resistant to further methylation above 30–32 % of methoxyl. These "resistant portions" form the subject of the present paper.

Further work by the author [1932, 2] on the acetates of the wood celluloses showed that the properties of the acetyl derivatives could be correlated with the proportion of resistant material contained in the polysaccharide. Increase in the amount of resistant material was accompanied by a corresponding increase in the specific rotation (in chloroform) in the dextro-sense. It was further shown that this resistance survived acetylation, de-acetylation and methylation, resulting in the formation of a soluble methylate of "depolymerised wood cellulose," thereby showing that mechanical properties (fibrous structure) or physical condition (high degree of polymerisation) were not in themselves factors inhibiting etherification, or producing resistance to hydrolysis.

From the first, work was hampered by an unavoidable lack of material but, as will be seen, a considerable amount of data regarding the "resistant portions" has been accumulated. Much of the evidence points to the "resistant portions" of different woods being chemically indistinguishable. Such a view, attractive as it seems, is put forward with every reservation on the grounds that we have here to deal with physical measurements relating to complex amorphous substances of unknown constitution.

It is now advantageous to detail the various steps involved in the investigation of the wood celluloses; the following scheme in the later stages (VI,

VII and VIII) could not, in every instance, be carried out, owing to insufficient quantities of material being available.



Stage III. Effect of the methylating reagents on the "resistant portions."

Repeated treatment with methyl sulphate and alkali, under varied conditions, resulted in only a small increase in the methoxyl contents of the resistant fractions. The products were white amorphous powders, insoluble in all solvents and showing no trace of organised structure, fibrous or otherwise, when examined microscopically (Table I).

Table I.

Source of cellulose	*Quercus pedunculata*	*Fagus sylvatica*	Thiriena pulp	Sitka spruce
"Resistant portion," yield %	40·1	23·9	22·5	19·1
"Resistant portion," OMe %	30·0	27·7	28·0	27·8
"Resistant portion," OMe % after methylation	32·2	31·5	30·3	29·6
Methylated cellulose, OMe %	38·8	39·1	39·0	37·3

Stage IV. Acetylation of the fully-methylated "resistant portions."

Subjection of the fully-methylated materials to acetylation by Barnett's method [1921] produced derivatives which displayed virtually identical analytical data and optical activities. The methoxyl contents were all slightly lower than was to be expected from the composition of the starting materials; a similar fact was observed by Denham and Woodhouse [1913] on acetylating

partially methylated cotton. The acetates were colourless, amorphous substances, easily soluble in all organic solvents except the paraffins; the solutions were clear and of inconsiderable viscosities. The specific rotations in chloroform were all identical, and the solids possessed closely similar melting-points. The apparent identity of these products is strongly emphasised by consideration of Table II.

Table II.

Source of "resistant portion"	*Quercus pedunculata*	*Fagus sylvatica*	Thiriena pulp	Sitka spruce
Acetate, yield %	89·6	89·0	84·1	95·7
,, OMe %	23·1	22·4	23·2	22·2
,, $COCH_3$ %	17·9	17·7	17·8	17·9
,, $[\alpha]_D$ in $CHCl_3$	+18·7°	+18·2°	+18·7°	+18·2°
,, M.P.	224–30°	226–30°	224–27°	230°

Calculated for $C_{10}H_{16}O_6$: OCH_3, 26·7 %
$COCH_3$, 18·3 %

Stage V. Cleavage of the acetyl derivatives.

Twenty-four hours' treatment at 120° with 2 % hydrogen chloride in methyl alcohol sufficed for complete hydrolysis of the acetates. The products were worked up in the manner described in a previous communication on wood cellulose [1932, 1]; the results of the experiments are detailed in Table III.

Table III.

Source of "resistant portion"	*Fagus sylvatica*	Thiriena pulp	Sitka spruce
Cleavage products isolated, %	78·8	88·3	70·0
Trimethyl-methylglucoside, %	31·1	32·9	32·4
Dimethyl-methylglucoside, %	44·2	44·1	55·5
Residual material, %	(accidentally lost)	10·5	4·1
,, OMe %	—	23·9	—

Calculated for $C_7H_{14}O_6$: OMe, 29·8 %.

It will be seen that the proportions of the different cleavage products show agreement in the different experiments. The increased amount of dimethyl-methylglucoside isolated from the Sitka spruce material is compensated by a corresponding decrease in the amount of residue. The latter was a hard glass and had a methoxyl content too low for monomethyl-methylglucoside, and may have been either unhydrolysed starting-material, or else impure monomethyl-methylglucoside.

Stage VI. The trimethyl-methylglucoside.

This portion of the cleavage products proved, in each case, to consist of 2:3:6-trimethyl-methylglucoside. No evidence of isomeric glucoses was found, and tetramethylglucose was present only in very minute traces. The occurrence of the latter compound need not be attributed to structural factors, for it is obvious that adventitious chains derived from the non-resistant fraction could remain associated with the resistant portions after the separation treatment.

Stage VII. The dimethyl-methylglucoside (material from Thiriena pulp).

The absence of substitution in the fourth hydroxyl group was proved by the fact that complete condensation took place in the cold between the free sugars and methyl alcohol containing hydrogen chloride. Employing the methods devised in this Laboratory by Rutherford [1932] and Oldham and Rutherford [1932] the amounts of 2:3-, 2:6-and 3:6-dimethyl-methylglucoside were determined in this portion of the cleavage products. The results were as follows:

2:3-Dimethyl-methylglucoside		4·4 %
3:6-	,, ,,	37·0 %
2:6-	,, ,,	58·6 %

Stage VIII. The residual material.

A portion of this material was found to distil at 190°/0·05 mm. After aqueous acid hydrolysis strong reduction of Fehling's solution was observed; the methoxyl content was 30·3 % (calculated for monomethyl-methylglucoside, 29·8 %). There is little doubt that the distillate was mainly this glucoside. The reducing solution was treated in the usual way with phenylhydrazine and crystals of an osazone-like substance were obtained, unfortunately in too small amount to permit of examination beyond that of melting-point determination. After recrystallisation, the crystals melted at 178° which is the melting-point of 6-monomethylglucosazone. The mixed melting-point with the latter substance showed no depression, while admixture with 3-monomethylglucosazone caused considerable lowering. A portion, therefore, of this residual material appears to consist of 6-monomethyl-methylglucoside.

Comparative experiments on partially methylated cotton.

Cotton cellulose methylated to the extent of 30·3 % of methoxyl was treated with Barnett's reagents under conditions identical with those of the acetylation of the "resistant portions." The insoluble material isolated amounted to 74·4 % of the calculated amount, had a methoxyl content of 16·7 % and showed $[\alpha]_D$ in chloroform, +10·0°. The material, therefore, differed markedly from that portion of the wood cellulose composing the "resistant portion." The noticeable loss of highly methylated material is in accordance with the observations of Denham quoted above.

The "resistant portion" of methylated depolymerised wood cellulose (Thiriena pulp).

Cleavage of the methylated depolymerised wood cellulose showed that, although the resistance to etherification had persisted, the resistant fraction no longer completely defied hydrolysis. The insoluble material isolated after 400 hours' treatment with 1 % hydrogen chloride in methyl alcohol had a methoxyl content of 29·9 % and exhaustive methylation raised this value to 32·3 %. The material was completely insoluble in chloroform, and after

acetylation a product apparently identical with the acetate of the "resistant portion" of the intact wood cellulose was obtained in 75·8 % yield. The analytical data of this material are compared with the corresponding values of the derivative of the depolymerised cellulose in Table IV.

Table IV.

Source of material	Intact cellulose	Depolymerised cellulose
"Resistant portion," yield %	22·5	14·0
,, ,, OMe %	28·0	29·9
Acetyl derivative, yield %	84·1	75·8
,, ,, OMe %	23·2	24·1
,, ,, $COCH_3$ %	17·8	16·9
$[\alpha]_D$ in chloroform of acetyl derivative	+18·7°	+18·9°
M.P. of acetyl derivative	224–227°	225–228°

As will be seen, apparently no significant change has occurred in the properties of the acetylated resistant fractions, although it must be admitted that the means of determining similarity of such substances are few and by no means reliable.

Experimental.

The general methods have been previously detailed in the other communications on wood cellulose.

Acetylation of the "resistant portions."

This was effected by Barnett's method, at a temperature of 50°. Solution of the methylated material in the reagents rapidly took place: the clear, yellow solution was poured into ten times its bulk of water containing a little sodium acetate, and the solid which separated collected on a filter and dried on filter-paper. The product was dissolved in chloroform, the solution dried over anhydrous sodium sulphate, filtered and the solvent evaporated under diminished pressure. The acetate was deposited as a colourless film, the properties of which have been detailed above. The relevant experimental data are given in Table V.

Table V.

Source of "resistant portion"	Wt. of material g.	Yield g.
Quercus pedunculata	2·175	2·379
Fagus sylvatica	1·880	1·942
Thiriena pulp	1·381	1·302
Sitka spruce	2·363	2·763
Depolymerised Thiriena pulp	0·514	0·476

Cleavage of the acetates of the "resistant portions."

The acetate (1 part) was heated for 24 hours with 2 % methyl alcoholic hydrogen chloride (25 parts) at 80°. Complete solution of the material rapidly took place. The reaction products were worked up as previously described. The data are given in Table VI.

Table VI.

Source of "resistant portion"	*Fagus sylvatica*	Thiriena pulp	Sitka spruce
Total cleavage products, g.	1·541	4·506	1·110
Trimethyl-methylglucoside, g.	0·485	1·481	0·360
Dimethyl-methylglucoside, g.	0·680	1·988	0·616
Residual material, g.	0·384	0·642	0·046
Wt. of acetate taken, g.	2·389	5·942	1·687

SUMMARY.

1. The "resistant portions" of wood celluloses cannot be methylated beyond a stage at which the composition of the methylate corresponds with that of a dimethyl-cellulose.

2. Acetyl derivatives of the "resistant portions" have been prepared. These derivatives from the different sources are soluble in a wide range of solvents and display a very close similarity in properties, strongly suggesting that they are identical. While the available data may lead to the conclusion that these acetates are one and the same substance, it does not follow that the "resistant portions," as they exist in the intact polysaccharide, are themselves identical.

3. The resistant material isolated on cleavage of fully methylated depolymerised wood cellulose displays properties very closely similar to those of the "resistant portion" of the corresponding intact methylated cellulose. This possibly indicates that Barnett's reagents effect depolymerisation until a definite level of complexity is attained, after which this action virtually ceases.

4. Cotton cellulose, methylated to 30 % of methoxyl, yields an acetyl derivative which displays no properties common to the acetates of the "resistant portions."

5. The cleavage products of the acetyl derivatives of the "resistant portions" have been shown to be a mixture of 2:3:6-trimethylglucoside, dimethyl-methylglucosides, and monomethyl-methylglucosides. In the case of Thiriena pulp cellulose the mixture of dimethyl-methylglucosides was shown to consist mainly of the 2:6- and 3:6- with a little of the 2:3-dimethyl-methylglucosides. Indication of the presence of 6-monomethyl-methyl-glucoside in the residual material was also obtained.

The author desires to place on record his indebtedness to Sir J. C. Irvine for the interest which he took in this work, and to the Carnegie Trust for the grant of a Senior Scholarship.

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LXXV. A METHOD FOR THE DETERMINATION OF LACTIC ACID PRODUCTION WITHOUT DESTRUCTION OF THE MUSCLE.

By COLIN ASHLEY MAWSON and ARTHUR DAVID RITCHIE.

(*Received March 19th, 1932.*)

Hill and Kupalov [1930] have shown that if a thin muscle such as a sartorius from *Rana temporaria* or a small *R. esculenta* is suspended in oxygen-free Ringer solution, and stimulated with not more than six single shocks per minute, the lactic acid diffuses out as fast as it is formed. In this way they were able to estimate the lactic acid formed in a series of about a thousand twitches by titrating the Ringer solution with alkali.

This method is evidently limited to experiments in which a large amount of lactic acid is formed: because (1) to prevent outward diffusion of phosphate from the muscle the Ringer solution must contain enough phosphate to cause considerable buffering; (2) there may always be some interchange of phosphate between muscle and Ringer solution which will alter the acid-base equilibrium; (3) there is some difficulty in eliminating errors due to loss of carbon dioxide from carbonates.

The ordinary method of estimating lactic acid by oxidation avoids all these difficulties. By adapting the method to deal with quantities of the order of 0·1 mg. it is possible to estimate the lactic acid produced in one hundred twitches of a pair of sartorii. The method has the advantage over all others, that the sample for estimation is removed without injury to the muscle. As the sample contains hardly anything but salt solution it can be oxidised directly without further treatment.

The principle errors in estimating small quantities of lactic acid by oxidation to aldehyde and iodimetric titration of the bound bisulphite are manifest in the form of large and variable values obtained in blank estimations. These can to some extent be avoided if, after steaming out the apparatus, an experiment is carried through using lactate solution before a blank estimation is done.

The method selected was to oxidise with freshly precipitated manganese dioxide in the presence of manganous sulphate and phosphoric acid [Friedemann and Kendall, 1929]. The flask is heated in a boiling water-bath and the acetaldehyde distilled over in a current of CO_2 [Tanaka and Endo, 1929]. The distillation apparatus is all glass, with one ground glass joint (see Fig. 1). The

method gives sufficiently low blank titrations for the purpose if the apparatus is kept thoroughly clean. The advantages of the method are considered to be:

(1) The rubber tubing and stopper for admitting CO_2 are not heated and do not come into contact with the reagents.

(2) There is no contamination with laboratory air.

(3) The process is carried out in the practical absence of oxygen.

(4) The apparatus is nowhere heated above 100°.

(5) The considerable reflux condensation retains substances of high boiling-point.

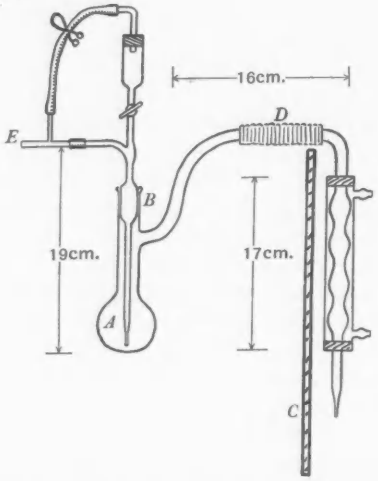

Fig. 1. *A*, 50 cc. flask; *B*, ground glass joint; *C*, asbestos shield; *D*, asbestos lagging; *E*, CO_2 inlet.
(*Made by the Scientific Glass-blowing Co., Manchester.*)

(6) On the other hand, as water is condensed before entering the receiver the aldehyde is already in solution before reaching the bisulphite solution.

(7) The volume of distillate can be controlled by variation of the rate of flow of CO_2.

Experimental.

A pair of sartorii was dissected out, leaving the bone attachment at both ends. The bones were trimmed down to the smallest convenient size and all other tissue cut away. A piece of silver wire was threaded through the two pieces of bone at the tibial end and another silver wire, passing through a small rubber stopper, hooked over the bone at the pelvic end. After soaking for half an hour in oxygenated Ringer solution the muscle was introduced into a narrow tube closed by the stopper at the lower end. The tube was clamped in a holder the height of which was adjustable by a screw, and the wire at the tibial end attached to an isometric tension lever. A measured quantity of the

Ringer solution to be used was introduced, the volume being adjusted so that the muscle would be just covered (4·0 cc. with the tube in use) when the sample extracted before twitches were commenced (1·0 cc.) had been removed.

The muscle was stimulated, by means of current supplied *via* the silver wires from a flashing neon lamp circuit. After the muscle had been subjected to any particular treatment a sample of the Ringer solution was removed, and the lactic acid estimated. For this estimation the apparatus was swept out with a current of CO_2, and a slow gas current allowed to continue. The flask was immersed in the water-bath and the delivery tube dipped into 2 cc. of dilute sodium bisulphite solution (1 cc. of saturated solution in 100 cc. of water) contained in a 25 cc. conical flask. The lactate solution was run into the flask and washed through with 2·5 cc. of manganous sulphate (2·0 %) and phosphoric acid (1·0 %) mixture. 5 cc. of manganese dioxide suspension (1 cc. of $N/10$ $KMnO_4$ + 9 cc. of $MnSO_4 - H_3PO_4$ mixture) were run slowly in, and the flask left for one minute. A fairly rapid current of CO_2 was then passed for 12 minutes, by which time a distillate of 4 to 5 cc. had collected. A drop of starch solution was added, and the excess bisulphite removed with $N/20$ iodine in 2 % KI solution, the final adjustment being made with $N/500$ iodine. 0·5 cc. of saturated sodium bicarbonate was then added and the liberated bisulphite titrated with $N/500$ iodine.

An end-point lasting at least 60 seconds after vigorous shaking must be obtained. A micro-burette is used with a fine tip dipping under the surface of the liquid. The iodine solution is standardised against a pure specimen of lithium lactate, and blanks are done before and after a series of experiments and the mean value used as the true blank.

The total lactic acid is calculated from the lactic acid found in the sample, on the assumption that the lactate is evenly distributed throughout the water present and that muscle contains 80 % water and bone 50 %. As the volume of Ringer solution added is large compared with that of muscle and bone, and as the sample removed for estimation is usually 90 % of the whole, the errors in the calculation are small, provided uniform distribution of lactate is actually obtained. Where, however, at the end of an experiment the whole of the fluid is to be used and made up to a known volume, the muscle is extracted with successive small portions of Ringer solution.

In the following series of experiments a known amount of lactate was added to a muscle and, after soaking for 10 minutes to allow diffusion to take

Exp. No.	Muscle No.	Mg lactic acid		
		Added	Observed	Error
98 *b*	1	0·037	0·036	−0·001
99 *c*	2	0·186	0·181	−0·005
101 *a*	4	0·075	0·081	+0·006
101 *d*	5	0·075	0·080	+0·005
103 *a*	6	0·071	0·069	−0·002
103 *b*	6	0·071	0·070	−0·001
104 *a*	7	0·071	0·064	−0·007
104 *b*	7	0·071	0·076	+0·005

place, the amount of lactate in the Ringer solution was estimated as described above. The muscle was kept on ice during the diffusion period to prevent formation of lactic acid due to resting metabolism.

Summary.

A method is described for the estimation of amounts of lactic acid of the order of 0·1 mg. without destruction of the muscle.

In conclusion, we wish to acknowledge a grant made to one of us (C. A. M). by the Medical Research Council.

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LXXVI. THE PREPARATION OF GONADOTROPIC HORMONES FROM NORMAL URINE AND URINE OF PREGNANCY.

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(*Received March 26th, 1932.*)

Zondek [1930] believes that apart from the growth hormone of Evans, three distinct anterior pituitary hormones are excreted in urine of pregnancy, a gonad-stimulating, a luteinising and a metabolism-stimulating principle. The last-named action may be partly due to the presence of the thyroid gland hormone. Recent work on the gland itself by Fevold *et al.* [1931] and others has corroborated Zondek's view on the separate existence of gonad-stimulating and luteinising principles. Urine, and particularly urine of pregnancy, shows the presence of many hormones, notably oestrin, placenta hormone, corpus luteum substances [Fevold *et al.*, 1932] *etc.*, often with similar chemical properties. This renders the chemical separation and purification particularly difficult, and great caution has to be exercised in attributing a certain activity in urine to the presence of anterior pituitary secretion. The gonad-stimulating substance behaves chemically like a polypeptide. The purity of such high molecular compounds is to be judged perhaps best by biological tests.

Testing the activity.

For the biological test we have used immature rats of both sexes just at the age when the sex can be clearly recognised. Animals which reach the weight of 25–35 g. after 5 daily injections are killed and the weight of seminal vesicles and uteri determined per g. of body weight. Controls of the same age and size gave values for the seminal vesicles of 0·15–0·2 mg. and for the uteri 1·5–2·0 mg. per g. of body weight. The opening of the vagina occurs on the sixth day of the experiment. For the actual assay we take only males, in which the weight of the organs can be determined more accurately, and we tentatively define the rat unit as the amount of hormone necessary to increase the weight of the seminal vesicles by 100 %, as compared with the controls. The dose is injected subcutaneously on 5 days. The female rats used in the same way give us indications regarding other hormonal admixtures. The accompanying Table shows a satisfactory response to increasing doses (the preparation used corresponded to 1–5 cc. of urine per day).

[1] With the technical assistance of Boris M. Viesnin.

Table I.

Dose cc.	Wt. of seminal vesicles in mg. per g. of body weight
0 (control)	0·21
1	0·25
2	0·60
3	1·23
4	1·64
5	2·00

Preparation of the active material and some of its properties.

The method of preparing the active material, used initially by Zondek, was the precipitation of the urine with alcohol and the extraction of the precipitate formed with water. Extracts prepared in this way are very crude and highly toxic to the test animals. Later Dickens [1930] described a method of salting out with ammonium sulphate. We prefer, however, the simple method to be described here, adopted after much experiment, which has rendered us good service for more than a year and a half. To the urine a solution of 30 g. per litre of quinine or benzoic acid, dissolved in the minimum volume of alcohol, is added in small portions with shaking. Both substances, and probably many others with similar properties, can be used successfully, the use of benzoic acid being now preferred. The next day the precipitate is separated and well washed with alcohol and ether. It is then extracted repeatedly with water and the resulting hormone solution purified by fractionation with alcohol of varying concentration, and finally by fractional evaporation and separation of impurities in vacuum at low temperature. The preparation of greatest purity was obtained by slow separation from 40 % alcohol, rubbing the vessel with a rod. The material under the microscope appears to be in a semi-crystalline state and showed the following activity. Five doses of 8γ each injected on 5 days (a total of 40γ) into rats caused a threefold increase in weight of seminal vesicles and twofold of the uteri, as compared with the controls. The yield was about 3 mg. per litre of urine of pregnancy. With purer preparations the opening of the vagina was delayed, compared with the effect of less purified material. The biological and chemical purity of the preparation is now under investigation, it retains most of its activity after preservation for a year in a dry form.

The gonad-stimulating principle is a colourless powder, partially soluble in 40 % alcohol and practically insoluble in 60 % alcohol. In contrast to insulin it is not precipitated by picric acid. The active material appears to be quite stable in urine; we have obtained an excellent preparation from pregnant urine kept for more than 2 years with traces of chloroform.

We have frequently used the benzoic acid method here described for the diagnosis of pregnancy with very good results, the main advantage being the lessened toxicity of the material to the test animals. By using this method we were able to demonstrate appreciable quantities of the gonad-stimulating principle in normal male and female urine. In these cases the material injected

corresponded to 150–200 cc. of urine. The results, however, were not constant and require reinvestigation.

The simultaneous administration of the gonad-stimulating substance and andronin (male sex hormone) to capons evoked less response than that of andronin alone, injections of the gonad-stimulating principle having no effect at all. Experiments with infantile rats were similar, combined administration showing less response than that of each substance separately.

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LXXVII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

X. THE EFFECT OF *ISO*BARBITURIC ACID ON THE EXCRETION OF NEUTRAL SULPHUR AND ETHEREAL SULPHATE.

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(*Received March 31st, 1932.*)

In the course of some work on the metabolism of pyrimidines Stekol and Cerecedo [1931] observed that a dog did not excrete any neutral sulphur in its urine after the administration of *iso*barbituric acid. They found that *iso*-dialuric acid and formyloxaluric acid had a similar effect. We have two reasons for distrusting these conclusions. Firstly, the complete elimination of neutral sulphur from the urine could only occur if the pyrimidine were altering the course of metabolism of a large number of different substances. It seems to us that such an effect is very unlikely. Secondly, Cerecedo himself [1930], in some earlier work on the metabolism of *iso*barbituric acid, did not find any such effect.

We have fed *iso*barbituric acid, in quantities similar to those used by Cerecedo, on six occasions and have been quite unable to verify the conclusions of Stekol and Cerecedo. These experiments were the first that had been carried out on one of the dogs (Ann) while the other dog (Patsy) had been used previously for metabolic work. It is possible that the effect observed by Stekol and Cerecedo was due to some abnormality in their dogs, the result of earlier metabolic work with other pyrimidines.

Experimental.

The technique used and the diet fed were the same as those described already from this laboratory [Hele, 1924; Hele and Pirie, 1931].

The results of the experiment on Patsy are shown graphically (Fig. 1). The two experiments on Ann (11 kg.) gave similar results. In the first of these a crude sample of *iso*barbituric acid was used, since it was possible that some impurity was the cause of the effect observed by Stekol and Cerecedo. In the second experiment with Ann, and in the four experiments with Patsy, pure *iso*barbituric acid, prepared by the method of Davidson and Baudisch [1925], was used.

It is clear from the figure that there is no diminution in the neutral sulphur after feeding *iso*barbituric acid. There is in fact a tendency for the neutral sulphur to rise, this may be due to the toxicity of the substance although it never seemed to cause any vomiting or loss of appetite. Our results are in

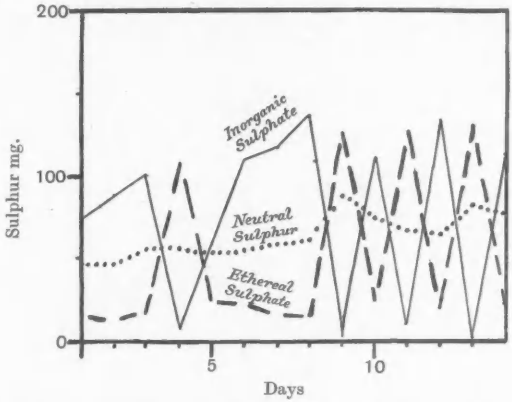

Fig. 1. Patsy, 8·2 kg. 2·5, 2·3, 2·2 and 3·5 g. of *iso*barbituric acid given orally on the 4th, 9th, 11th and 13th day.

agreement with those of Stekol and Cerecedo in showing that *iso*barbituric acid is very readily conjugated with sulphate by the dog.

Summary.

*iso*Barbituric acid does not cause the disappearance of the neutral sulphur fraction from a dog's urine.

It is readily conjugated with sulphate.

The expenses of this work were in part covered by a grant to Dr T. S. Hele from the Government Grant Committee of the Royal Society.

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LXXVIII. THE INFLUENCE OF GLUTATHIONE ON REDUCTION AND ROTATION OF BLOOD-FILTRATES.

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During the last decade numerous reduction methods have been published for the determination of blood-sugar. The literature on the optical rotation of protein-free filtrates of blood is less extensive. The majority of investigators in this field have determined the rotation after concentration of the blood-filtrates *in vacuo*. During this procedure which takes a fairly long time and is accompanied by increase of acidity, the possibility of hydrolysis of complex molecules with production of optically active bodies is obvious. Moreover a loss of reducing substances during concentration has been observed [Opler, 1910, 1911; Stepp, 1919; Barenscheen *et al.*, 1926]. Some workers have used dialysates, but the results obtained in this way can be applied to plasma only. It seemed therefore worth while to adapt a method for micropolarisation that had been worked out by Meyer [1930] to the determination of the rotation of protein-free blood-filtrates. For technical details the reader is referred to the original publication. The principle of the method is the photometric measurement of the intensity of light, penetrating a solution of an optically active substance, placed between crossed Nicols. The method permits of the determination of 0·01 % solutions of glucose to within 5 %; its accuracy is not affected by the presence of the Folin-Wu protein precipitants or by variations in p_H from 3·5 to 7·0.

Reduction and rotation.

Hedon [1898] was the first to point out that the rotation method always yielded lower results for blood-sugar than reduction methods. This fundamental observation has been confirmed by nearly all subsequent investigators who have used the greatest variety of ways of protein precipitation, reduction, concentration of the filtrates, *etc.* The discrepancy between reduction and rotation was only denied by Opler [1910, 1911], Takahashi [1911], Griesbach and Stützer [1913] and Maase and Tachau [1915], who used respectively phosphotungstic acid, colloidal iron hydroxide and mercuric chloride with hydrochloric acid as protein-precipitating agents, a fact to which we shall refer later on.

Most of these older investigators ascribed the discrepancy between the two methods to the presence of non-sugar reducing substances in the blood; this

seemed the more probable since Stepp [1919, 1922] demonstrated that the "sugar values" obtained by rotation agreed much better if they were not compared with apparent reduction figures, but with the amount of true fermentable sugar present in the blood.

Winter and Smith [1923, 1, 2, 3, 1924] tried to explain the discrepancy between reduction and rotation by assuming that the sugar in normal blood was γ-glucose, whilst in diabetic blood, in which they observed no discrepancy, it was α, β-glucose. Their conclusions have been questioned by van Creveld [1923], Denis and Hume [1924], Foster [1923], Hewitt [1923], Laquer [1925], MacLeod [1924], Mozotowski [1924], Thannhauser and Jenke [1924], and Vischer [1926].

A few years later Lundsgaard and Holbøll [1924, 1925] suggested the existence in blood of a "new glucose" on the basis of the observed discrepancy between reduction and rotation in dialysates of blood-plasma. Barbour [1926] and Anderson and Carruthers [1926] who repeated their work have not followed them in their conclusions.

Wright, Herr and Paul [1928] have also compared reduction and rotation of protein-free dialysates of blood-plasma. They found but a small discrepancy between both methods if the blood was fresh and suggested that the difference might be partly due to the presence of lactic acid in the plasma. It appeared that the discrepancy increased while glycolysis proceeded, the initial values showing hardly any difference at all; furthermore they showed that after acidification of the dialysates the low rotation values increased until they corresponded almost exactly to the reduction figure. As the sodium lactate from the blood is laevorotatory and lactic acid itself dextrorotatory but much less active, this phenomenon would fit in very well with their explanation. However, as these workers themselves have pointed out, the quantity of lactic acid present under normal circumstances in the blood (20 to 30 mg. per 100 cc.) is far too small to account for the whole of the difference observed. Furthermore if one uses not plasma but whole blood for these comparisons a difference is found which is not changed at all by addition of acid, so that lactates can account for but a small part of the discrepancy.

In this paper evidence is brought forward to support a quite different explanation of the low polarisation values of protein-free filtrates, based on the presence of non-sugar reducing substances in the blood. Recent work by Somogyi [1927, 1928] and others has considerably extended our knowledge of these reducing substances. Their influence on various blood-sugar methods depends on their behaviour towards every special reducing agent as compared with glucose [Herbert and Groen, 1929; Somogyi and Kramer, 1928]. Their chemical nature has been studied by Benedict and Newton [1929], Herbert, Cotonio-Bourne and Groen [1930] and Herbert and Cotonio-Bourne [1930].

Uric acid, creatine, creatinine and ergothioneine can be dismissed as possible causes of the residual reduction, as normally these substances occur in far too small amounts to have any appreciable effects on blood-sugar methods.

The paramount importance of glutathione in this respect has been specially emphasised by Herbert and Cotonio-Bourne [1930] who regarded glutathione as the only substance responsible for the phenomenon. They pointed out that the magnitude of the non-glucose reducing fraction as determined by various methods corresponded almost exactly with the results of a calculation based on the assumption that the normal glutathione content of the blood is about 52 mg./100 cc. The fact that these saccharoids (the name given by Benedict [1931] to the reducing non-sugars in the blood) are chiefly confined to the corpuscles formed another of their arguments.

In a recent paper Benedict [1931] has subjected Herbert and Cotonio-Bourne's postulate to a vigorous criticism. The actual amount of glutathione in the blood is probably less than 52 mg./100 cc. and the non-sugar reducing fraction of the plasma, small as it may be, cannot consist of glutathione, which substance is confined exclusively to the corpuscles. In the present state of affairs therefore glutathione cannot be regarded as the only reducing non-sugar of the blood, but to our mind there can be little doubt that it is by far the most important saccharoid. This is also in accord with Benedict and Newton's [1929] former conception.

The idea that glutathione is responsible for the long known discrepancy between blood-sugar estimations by rotation and reduction was originally suggested by Holden [1925], and formed the starting-point of the present investigation.

Experimental.

For the first series of experiments we used blood of normal adults, convalescent patients or of patients with local affections who did not show evidence of any metabolic disturbance. Blood was taken fasting, after ordinary meals, or after administration of glucose by mouth; none of these factors appeared to influence the results. Clotting was prevented by addition of oxalate (20 mg. per 10 cc. of blood), or by defibrination; immediately after the collection of the blood the protein was precipitated with sodium tungstate and sulphuric acid after Folin and Wu, centrifuged and the supernatant fluid filtered through Jena sintered glass funnels (No. 17-G-4). This extra filtration is necessary to obtain absolutely clear filtrates for the photometric polarisation method. As reduction method the Somogyi [1926] modification of the Shaffer-Hartmann technique was used throughout. All estimations were carried out in duplicate; these duplicates never showed a difference exceeding 5 mg./100 cc. As standard solution for the polarimetric determination two glucose solutions were used, containing respectively 10 and 20 mg. glucose per 100 cc. corresponding to a glucose content of 100 and 200 mg./100 cc. in the undiluted blood. These standard solutions were made up fresh every time by dilution from a 1 % stock solution, to avoid errors due to mutarotation. The results of this series of simple comparisons between rotation and reduction are shown in Table I.

In every instance we found a considerably lower value by rotation than by

Table I. *Glucose in mg./100 cc. of blood.*

Experiment	Reduction	Rotation	Difference
1	89	40	49
2	71	50	21
3	92	70	22
4	82	50	32
5	82	46	36
6	103	74	29
7	86	50	36
8	118	84	34
9	81	50	31
10	106	67	39
11	114	64	50
12	74	42	32
13	118	90	28
14	114	70	44
15	112	64	48
16	107	75	32
17	113	88	25
18	97	63	34

reduction, the difference in Table I showing a maximum of 50, minimum of 21 and an average of 34 mg./100 cc. expressed as glucose.

A second series of experiments was carried out in the blood and plasma simultaneously. Glutathione is present in the corpuscles only and if it were the cause of the discrepancy between physical and chemical methods one might expect both methods to agree, if applied to plasma. Table II shows that this

Table II. *Glucose expressed in mg./100 cc.*

Blood			Plasma		
Reduction	Rotation	Difference	Reduction	Rotation	Difference
116	82	34	110	114	−4
85	59	26	81	79	2
82	59	23	78	77	1
135	111	24	131	139	−8
107	80	27	103	106	−3
			97	100	−3
			97	90	7
			94	100	−6
			83	79	4

is actually the case. For plasma precipitation only half the quantities of sodium tungstate and sulphuric acid of those used for blood were added; in a few cases we used plasma-ultrafiltrate, but the final dilution was always 1 : 10.

The samples of blood in this series showed differences between rotation and reduction varying between a minimum of 23 and a maximum of 34, the average difference being 27 mg./100 cc., which may be called in fairly good agreement with the results of Table I. For the plasma, maximum, minimum and average differences are respectively − 8, + 7, − 1, which shows that the cause of the observed discrepancy is present in the corpuscles only. This result in itself renders the hypothesis of a γ- or "new glucose" extremely improbable, as it is difficult to imagine how a substance like glucose, which can diffuse freely between corpuscles and plasma, could be present in the usual α, β-form

in the plasma and in an isomeric state in the corpuscles. It also makes it very improbable that lactic acid is responsible, as this substance is also equally divided between corpuscles and plasma.

A third series of determinations was carried out in blood-filtrates, which had been obtained by the so-called "sulphate precipitation technique" [Herbert and Cotonio-Bourne, 1930] in which haemolysis of the corpuscles does not take place and glutathione does not pass out into the surrounding fluid. In Table III we have compiled our results of a threefold comparison

Table III. *Glucose in mg./100 cc.*

Blood: "Folin and Wu"			Blood: "Sulphate technique"			Plasma		
Reduction	Rotation	Difference	Reduction	Rotation	Difference	Reduction	Rotation	Difference
82	63	19	73	88	−15	87	84	3
74	50	24	65	77	−12	76	74	2
81	52	29	69	80	−11	84	79	5
87	58	29	65	70	− 5	—	—	—
93	61	32	77	81	− 4	84	79	5
102	50	52	82	87	− 5	99	103	− 4
92	58	34	81	88	− 7	88	98	−10
82	47	35	67	72	− 5	79	84	− 5
98	60	38	80	86	− 6	96	94	2
90	65	25	72	75	− 3	88	96	− 8
87	59	28	71	80	− 9	84	80	4
119	96	23	97	102	− 5	113	108	5

between rotation and reduction as carried out in the blood, treated both with the ordinary Folin and Wu precipitation and with the Herbert and Bourne method, and in the plasma.

The Table shows that if one prevents glutathione from passing into the filtrate a much better agreement between rotation and reduction is obtained than in the case of the ordinary Folin and Wu filtrates. The differences for whole blood are by the Folin and Wu method minimum 19, maximum 52, average 31, and for the plasma minimum − 10, maximum + 5, average 0, mg./100 cc., which confirms the results of the preceding Tables. For the "sulphate technique" the differences vary between − 12 and − 3, showing an average of − 7 mg./100 cc. The agreement in this case is not so perfect as found in the plasma, but, as the magnitude of the differences is hardly outside the limit of experimental error, we are inclined to attach more value to the gross agreement than to small discrepancies. In this state of the work the good agreement obtained between results of reduction and rotation by Opler, Takahashi, Griesbach and Stützer, and Maase and Tachau became clear. These investigators used for the protein precipitation respectively iron hydroxide, phosphotungstic acid and mercuric chloride with hydrochloric acid, which reagents are all known to precipitate or destroy reduced glutathione; the same holds true for uranyl acetate of which Vischer claimed that it was able to yield a protein-free filtrate in which polarisation and rotation agreed.

Reduction and rotation of glutathione.

Finally we have used our figures for a calculation in support of the view that glutathione can explain the different results of chemical and physical methods. The glutathione content of normal human blood is still under discussion; the majority of investigators give values for reduced glutathione between 30 and 50 mg./100 cc. of blood. The amount of this substance varies with the corpuscle volûme of the blood, but as the haemoglobin contents of our subjects were about normal, we are justified in leaving out this factor.

The results obtained by us by various methods have led us to regard 40 mg./100 cc. as the average figure for the glutathione of normal human blood.

The glucose equivalent of a certain quantity of glutathione, as determined by various blood-sugar methods, has been worked out by Herbert, Cotonio-Bourne and Groen [1930]. For the Shaffer-Hartmann method they found that 100 mg./100 cc. of glutathione yield a reduction equivalent to 39 mg./100 cc. of glucose. 40 mg./100 cc. of glutathione present in human blood would therefore raise the true sugar figure by 16 mg./100 cc., a value somewhat lower than that actually found for the reducing non-sugars, the remaining 6 mg./100 cc. being caused by less important constituents of the blood.

What will be the influence of 40 mg./100 cc. of glutathione on the rotation of a protein-free blood-filtrate? In the literature on glutathione, we could find for its rotation only two exact figures. For the mercury green light the specific rotation of reduced glutathione amounts according to Hopkins [1929] to $-18{\cdot}5°$, and according to Mason [1930] to $-20°$. With samples of glutathione, obtained from the Eastman Kodak Co. and from the Hoffmann La Roche Co. we found for the filtered yellow light, used in our experiments, a specific rotation of $-17{\cdot}5°$; this figure was determined with an ordinary Lippich polarimeter. We also determined the specific rotation of glutathione with the photometric micropolarisation method. As glutathione is laevorotatory we used fructose of high purity for comparison; in this way one finds the rotation equivalent of glutathione compared with fructose and, as the specific rotation of the sample of fructose used by us was $-90°$, we were able to calculate the specific rotation of glutathione. We also measured the rotation of glucose solutions of known strength, to which varying quantities of glutathione had been added; the decrease in rotation of the glucose solutions was used for the calculation of the specific rotation of glutathione.

The results are laid down in Tables IV and V.

Table IV. *Rotation of glutathione compared with fructose.*

Exp. No.										
1	20	mg./100 cc.	GSH	rotation	equivalent	to	4·00	mg./100 cc.	fructose :	$[\alpha]_D$ −18·0
2	30	,,	,,	,,	,,		6·00	,,	,, :	−18·0
3	40	,,	,,	,,	,,		8·40	,,	,, :	−18·9
4	10	,,	,,	,,	,,		2·34	,,	,, :	−21·0
5	20	,,	,,	,,	,,		4·55	,,	,, :	−20·5
6	10	,,	,,	,,	,,		1·99	,,	,, :	−17·9
7	30	,,	,,	,,	,,		6·72	,,	,, :	−20·2

Table V. *Rotation of glutathione compared with glucose.*

Exp. No.										
1	8 mg./100 cc. GSH rotation equivalent to 2·7 mg./100 cc. glucose : $[\alpha]_D$									−17·1
2	8	,,	,,	,,	,,	2·6	,,	,,	:	−17·7
3	40	,,	,,	,,	,,	16·0	,,	,,	:	−21·0
4	60	,,	,,	,,	,,	24·0	,,	,,	:	−21·0

The average specific rotation of glutathione calculated from the figures in these Tables is −19·2°, which is in sufficient agreement with the optically determined rotation of much stronger solutions, to demonstrate the reliability of the method. If we use the optically determined figure −17·5° as the basis of our calculation, we find that 100 mg. of glutathione show a rotation of opposite sense and equivalent magnitude to 33 mg. of glucose, and a glutathione content of 40 mg./100 cc. in the blood would therefore diminish the polarisation figure of the true sugar in the blood by about 13 mg. The reduction figure being 16 mg. too high, the polarisation figure 13 mg. too low, the difference between the two methods would be 29 mg./100 cc. of glucose. Actually the average observed figure, calculated from the results in Tables I, II and III, for the difference between reduction and rotation was 32 mg./100 cc. If the experimental errors of both methods and the physiological variations in the glutathione content of the blood are taken into consideration, the agreement between observed and calculated figures is remarkable and in itself a strong argument in favour of the important rôle played by glutathione in both reduction and rotation estimations.

Diabetes.

The behaviour of diabetic blood has been studied in a series of experiments, in which the ordinary Folin and Wu method, the "sulphate technique" for precipitation of the protein and the plasma values were compared. The experiments were carried out on fasting patients, after various meals, with and without insulin administration. The results are tabulated in Table VI and show that all these factors do not influence the behaviour of diabetic blood, which in this respect yields the same results as that of normal people.

Table VI. *Diabetes.*

Glucose in mg./100 cc.

	Blood: "Folin and Wu"			Blood: "Sulphate technique"			Plasma			
Name	Reduction	Rotation	Difference	Reduction	Rotation	Difference	Reduction	Rotation	Difference	Remarks
M.	218	167	51	—	—	—	—	—	—	Coma
M. H.	290	247	43	—	—	—	—	—	—	No insulin
d L.	137	114	23	—	—	—	133	132	+1	No insulin
S. A.	246	218	28	—	—	—	259	253	+6	8 hrs. after 10 units insulin
S. B.	243	216	27	—	—	—	256	259	−3	No insulin
F.	92	71	21	80	82	−2	—	—	—	Fasting, no insulin
R.	276	246	30	259	259	0	—	—	—	1 hr. after 20 units insulin
B. G.	231	201	30	208	212	−2	—	—	—	No insulin
G.	221	197	24	200	201	−1	—	—	—	No insulin
R.	155	126	29	131	128	+4	160	157	+3	½ hr. after 30 units insulin
V.	282	251	31	254	255	−1	304	300	+4	4 hrs. after 20 units insulin
P.	196	162	34	175	179	−4	204	200	+4	8 hrs. after 15 units insulin
B.	113	80	33	103	103	0	112	106	+6	8 hrs. after 10 units insulin

The bloods in this Table show differences varying from a minimum of 21 to a maximum of 51, on the average 31 mg./100 cc.; the plasma shows a difference between both methods of maximum + 6, minimum − 3, average 3; for the "sulphate technique" these differences are maximum + 4, minimum − 4, average − 1 mg./100 cc.

We have often left our filtrates to stand overnight at room temperature and carried out experiments after 24 hours. We were never able to find a rise in rotation as described by Winter and Smith [1923, 1, 2, 3]. Unfortunately we could not succeed in keeping the filtrates sterile for more than 24 hours; after prolonged standing faint turbidities appeared, which would have been no obstacle for any ordinary optic polarimetric estimation, but rendered the application of the photometric technique impossible. We cannot exclude therefore the possibility of an increase in rotation of the blood filtrates after prolonged standing. But as glutathione is liable to be decomposed if the medium does not show a marked acid reaction, it is quite possible that the disappearance of this laevorotatory substance from the solution may have caused the rise in rotation observed by Winter and Smith. In any case the phenomenon in itself is not sufficient to uphold the theory of the presence of γ-glucose.

In his latest publication Winter [1930] has succeeded in isolating the glucose from human blood; he identified it as the α-form; no evidence of a separate isomeric modification was found.

Summary.

1. A method is described for the determination of very small rotations in non-concentrated protein-free filtrates of blood and plasma.

2. In Folin and Wu filtrates of normal human blood the difference between reduction, estimated with the Shaffer-Hartmann-Somogyi method, and the rotation amounts on the average to 32 mg./100 cc. expressed as glucose.

3. Plasma filtrates show almost complete agreement between the results of reduction and rotation.

4. In filtrates of blood prepared with the "sulphate technique" of Herbert and Bourne, reduction and rotation methods agree within the limits of experimental error.

5. The reduced glutathione present in the blood is the main cause of the differences between the results of reduction and rotation methods. The absence of this substance from plasma and "sulphate filtrates" explains the agreement mentioned under 3 and 4.

6. 100 mg. of glutathione show a reduction equivalent to 39 mg. of glucose and a rotation equivalent to 33 mg. of glucose, but oppositely directed. If the glutathione content of human blood amounts to about 40 mg./100 cc., a discrepancy of 29 mg. glucose per 100 cc. between reduction and rotation may be expected, which is in agreement with the observed value of 32 mg./100 cc. The influence of some less important substances (lactates among them) affords

a satisfactory explanation of the small discrepancy between the observed and calculated figures.

7. The blood of diabetic patients does not differ from normal blood, as far as the differences between reduction and rotation are concerned.

8. The hypothesis of a γ-glucose or "new glucose" for the explanation of the differences studied in this paper is unnecessary and untenable.

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LXXIX. THE PHYSICO-CHEMICAL BEHAVIOUR OF LECITHIN.

II. THE CAPILLARY ACTIVITY OF LECITHIN-CHOLESTEROL DISPERSIONS.

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(*Received April 19th, 1932.*)

The work described in the present paper is a continuation of that of Price and Lewis [1929] who measured, by means of the Sugden maximum bubble pressure method (a dynamic method), the surface tension of dispersions in water of lecithin (prepared and purified by the method of Levene and Rolf [1927]) as a function of the p_H of the system. It was found that a maximum value of the surface tension occurred at p_H 2·6 (approx.), a position which agrees well with the value 2·7 for the isoelectric point obtained by an entirely different method by Fugii [1923–24]. The surface tension fell to a very marked minimum on either side of the maximum referred to.

Employing the same technique and methods of preparation, the above results have been repeated and confirmed by the present authors. With this as a starting point the next step was to investigate what modification, if any, was produced by the presence of cholesterol in the lecithin dispersion. It was found, as might have been expected, that pure cholesterol owing to its insolubility and lack of dispersibility in water exerted no effect of itself upon the surface tension of water. This was confirmed by a series of measurements in which cholesterol was first dissolved in ether, the solution being poured into water. It was found that the capillary effect was due entirely to the ether.

Before dealing with the behaviour of lecithin-cholesterol dispersions, however, it is convenient to give the experimental evidence which is concerned with the dimensions of the capillary-active unit itself.

The capillary-active unit in lecithin dispersions.

From the fact that lecithin dispersions are visibly turbid it follows that the disperse units are, for the most part at least, of very considerable size, *i.e.* of the order of 10^{-4}–10^{-5} cm. radius. It is a point of some theoretical interest to know whether units of this order are responsible for the capillary effects produced or whether the latter are due to the presence of much smaller units either of truly colloidal or even molecular magnitude. To determine this a series of

filtration experiments was carried out (*a*) with a coarse filter; (*b*) with an ultrafilter (collodion).

Series (*a*). The filter was a good commerical preserve paper. The filtrate was itself turbid but less so than the original. In this case any colloidal and molecular units would come through with ease and if these were the truly capillary-active constituents the surface tension should be unchanged. Actually the filtrate possessed somewhat higher tension than the original, *i.e.* the filtrate approximated more nearly to pure water, indicating that the large units are certainly capillary-active though this of itself does not exclude the presence of active units of small size. For the sake of comparison, filtration was carried out with sucrose solution, the concentration of which was found to be unaffected by this filter, as is shown by the constancy in the optical rotation. The experimental observations are as follows.

Sucrose solution (20 °/$_o$):

Angle of rotation { before filtration = 47·53°, of filtrate = 47·33°.

Lecithin (1 °/$_o$ dispersion):

Before filtration, surface tension = 32·0 dynes/cm.
Surface tension of filtrate = 51·8 dynes/cm.

Lecithin (0·5 °/$_o$ dispersion):

Before filtration, surface tension = 66·2 dynes/cm.
Surface tension of filtrate = 69·4 dynes/cm.

Series (*b*). In this case a collodion ultrafilter was employed. The filtrate now came through water-clear. As the results show, the surface tension of the filtrate was practically that of water itself. At the same time sucrose molecules, as would be expected, passed through with ease. The results are as follows.

Sucrose solution (20 °/$_o$):

Angle of rotation { before filtration = 47·54°, of filtrate = 47·30°.

Lecithin (0·5 °/$_o$ dispersion):

Before filtration, surface tension = 66·2 dynes/cm.
Surface tension of filtrate = 71·0 dynes/cm.
(Surface tension of pure water = 71·78 dynes/cm.)

The results of both series of measurements demonstrate that there are no capillary-active units of molecular or colloidal size in a lecithin dispersion, the capillary effect being due entirely to the large units which give rise to the turbidity.

Preparation of lecithin-cholesterol dispersions.

The desired amounts of pure lecithin and cholesterol were dissolved in a small volume of specially purified ether and the solution was evaporated to dryness. The residue was redissolved in ether and again evaporated. This was repeated two or three times, the evaporation being carried out under reduced

pressure and at low temperature to avoid decomposition of the lecithin. The final dry residue was shaken with the correct quantity of water and the resulting dispersion kept in an atmosphere of nitrogen.

Except in the case of the dispersion which contained the highest amount of cholesterol (namely, lecithin : cholesterol = 1 : 3) all the dispersions prepared were very stable, no precipitation occurring. In the one exceptional case not all the cholesterol was combined with or peptised by the lecithin, with the result that there was a certain amount of solid cholesterol present as a precipitate. (It was demonstrated that this was pure cholesterol.) It is evident that the ratio recorded as 1/3 is in fact slightly larger than this.

Experimental results.

All measurements were carried out at 25°. As in the work of Price and Lewis, the dynamic surface tension was found to be independent of the rate of bubble-formation, which could be varied, in the apparatus employed, between 1 bubble per 0·5 sec. and 1 bubble per 3 secs. The p_H determinations were carried out by means of the gold-quinhydrone electrode as well as by the glass electrode with valve. The composition of the dispersed material is expressed in grams of lecithin to grams of cholesterol, *e.g.* 4 : 1. Different concentrations of any given lecithin-cholesterol dispersion were employed, *e.g.* 0·5 %, that is 0·5 g. of dispersed material per 100 cc. of aqueous "solution." The surface tension of water was taken as 71·78 dynes per cm.

In each case the surface tension was determined at a series of different p_H values covering a wide range of p_H. These arbitrarily chosen p_H values however differed from case to case. In order to make the results more readily comparable for dispersions of different chemical composition the actual values have been graphed and, from the resulting curves, the values of surface tension read off at a series of p_H values the same for all the dispersions for which the data are recorded. The results are recorded in the following Tables which include besides the numerical data certain comments necessary to make them self-explanatory.

Table I. *0·5 % dispersion of lecithin.*

p_H		Surface tension (dynes/cm.)	
0·50		58·0	
1·00		44·2	
1·20		40·8	1st minimum
1·50		47·0	
2·00		65·8	
2·50		68·9	
2·60	Isoelectric point	69·1	Maximum
3·00		66·6	
3·50		56·2	
3·70		55·7	2nd minimum
4·00		60·0	
4·50		66·4	
5·00		66·7	
5·50		66·7	Flat portion parallel
6·00		66·7	to p_H axis
6·50		66·7	

Table II. *0·5 % dispersion.*

Ratio: lecithin/cholesterol = 4/1.

p_H		Surface tension (dynes/cm.)	
0·50		67·6	
1·00		69·2	
1·25		69·6	1st maximum
1·50		69·20	
2·00		67·20	
2·50		61·0	
2·70		60·4	Minimum
3·00		62·8	
3·50		68·0	
4·00	Isoelectric point ?	70·2	2nd maximum
4·50		67·6	Very shallow minimum
5·00		68·0	
5·50		68·2	
6·00		68·1	Flat portion
6·50		68·0	
7·00		68·0	

Table III. *0·5 % dispersion.*

Ratio: lecithin/cholesterol = 2/1.

p_H		Surface tension (dynes/cm.)	
1·00		64·2	
1·35		66·0	1st maximum
1·50		65·7	
2·00		63·0	Minimum
2·50		64·8	
3·00		67·6	
3·50		69·6	
4·00	Isoelectric point ?	70·8	2nd maximum
4·50		70·2	
5·00		69·8	
5·50		69·7	
6·00		69·5	Flat portion
6·50		69·6	

Table IV. *1·0 % dispersion.*

Ratio: lecithin/cholesterol = 1/1.

p_H		Surface tension (dynes/cm.)	
1·00		65·4	
1·50		65·0	
2·00		63·6	
2·35		62·4	Minimum
2·50		62·5	
3·00		64·8	
3·50		66·4	
4·00		67·8	
4·25	Isoelectric point ?	68·1	Maximum
4·50		67·8	
5·00		67·2	
5·50		67·3	
6·00		67·6	
6·50		67·5	Flat portion
7·00		67·3	

The concentration of the dispersion is in this case 1 %, *i.e.* 0·5 % with respect to lecithin. The values are thus directly comparable with those of Table I for lecithin alone.

Table V. *0·5 % dispersion.*

Lecithin/cholesterol = 1/3 (approximately).

p_H		Surface tension (dynes/cm.)	
0·50		65·4	
1·00		68·4	
1·50		69·4	1st maximum
2·00		68·2	
2·50		67·0	Minimum
3·00		68·2	
3·50		70·8	
4·00	Isoelectric point ?	71·6	2nd maximum
4·50		71·2	
5·00		70·7	
5·50		70·7	Flat portion
6·00		70·9	
6·50		70·9	

At this concentration some free cholesterol separates out.

Inspection of the above Tables shows that addition of cholesterol, whilst leaving the general capillary behaviour of lecithin as a function of p_H unchanged as a whole, nevertheless introduces modifications in the magnitude of the surface tension values and at the same time appears to alter the location of the maxima and minima exhibited by dispersions of the pure substance. The most important modification consists in what appears to be a shift in the position of the isoelectric point (as determined by the central maximum surface tension) from p_H 2·6 to p_H 4. This very great shift into the region of higher p_H values is brought about even in the case of that dispersion in which the lecithin is still in great excess, namely, 4 : 1. In view of the fact that in blood-serum the ratio of lecithin to cholesterol is about 1 : 1 and of the order 5 : 1 in normal tissue of various kinds [Jowett, 1931] it follows that the significant reaction which would determine the electrical behaviour of the naturally occurring (impure) material would be p_H 4. Whether this is modified further by the presence of protein is a matter now under investigation.

Summary.

By means of the Sugden maximum bubble pressure method (a dynamic method) the surface tensions of dispersions containing lecithin and cholesterol in different relative amounts have been determined as a function of p_H. The position of the isoelectric point (identified with that of the central surface tension maximum) is altered by the addition of cholesterol from the value p_H 2·6, characteristic of pure lecithin, to p_H 4.

It is shown that the capillary-active units are the large particles which give turbidity to the dispersion, no evidence being obtained for the existence of capillary-active units of colloidal (or molecular) dimensions.

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LXXX. NOTE ON THE CAPILLARY BEHAVIOUR OF GLYCINE.

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(*Received April 19th, 1932.*)

In view of the capillary activity exhibited by dispersions of lecithin in water [Price and Lewis, 1929] and of lecithin-cholesterol dispersions [Jones and Lewis, 1932] it was of interest to see whether a maximum in the surface tension would likewise occur at the isoelectric point in the case of a simple amphoteric substance such as glycine which does not exhibit any colloidal or emulsoidal properties but dissolves in water in the form of simple and double molecules [Lewis, 1931].

The method of measurement, namely, the dynamic method of Sugden, which had been employed in the case of lecithin dispersions, was again used in the present case. The temperature was 25°. The results obtained for the variation of surface tension with p_H in the case of two concentrations of glycine, namely, 10 % and 5 %, are given in Fig. 1. Inspection of the curves indicates

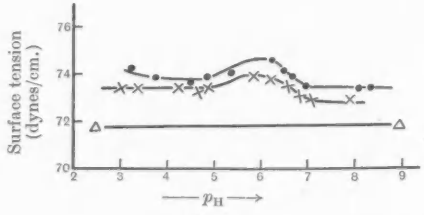

Fig. 1.

—●—●— = 10 % solution of glycine in water.
—×—×— = 5 % " " "
△——△ = Pure water.

that once more the surface tension is maximal at or close to the known isoelectric point of glycine, namely, p_H 6·08. At the same time glycine differs from lecithin in that the surface tension over the p_H range examined is greater than that of water. It follows that the explanation suggested by Price and Lewis to account for the maximum in the case of lecithin requires modification. This explanation regarded the capillary-active units as dipoles, each carrying the

same amount of positive and negative electricity, the dipoles being arranged vertically to the surface with positive and negative poles alternating. It was assumed that, at the isoelectric point, the contiguous dipoles annulled each other's effect upon the surface tension so that the value tended to approximate to, but could not exceed, that of water itself. In the light of the glycine results it is necessary to assume that in the isoelectric condition the dipoles are able to increase the surface tension even above that possessed by water. Whether the observed tension is greater or less than that of water will thus depend upon the nature of the material employed. The remaining treatment of the lecithin case, to account for the decrease in surface tension on either side of the isoelectric point, appears to hold good generally.

It may be mentioned that the influence of addition of NaCl (0·1 *N*) to solutions containing 10 % glycine was determined over the p_H range 2·68 to 8·80. The values lay approximately on a horizontal line parallel with the water line itself the average surface tension being approximately one dyne greater than that of water. The presence of the ions of the simple inorganic salt thus serves to obliterate any appreciable effect due to the glycine. Since glycine and sodium chloride raise the surface tension, both are negatively adsorbed at the surface but it appears that the glycine is more completely removed than is the sodium chloride.

SUMMARY.

Using the Sugden bubble method it is shown that the surface tension of glycine dissolved in water has a maximum value at the isoelectric point (p_H 6·08).

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LXXXI. THE FORMATION OF ENTEROKINASE FROM A PRECURSOR IN THE PANCREAS.

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(*Received March 15th, 1932.*)

HEIDENHAIN [1875] first observed that fresh extracts of the pancreas gland have no measurable tryptic activity, but that on keeping "spontaneous activation" occurs and the extracts develop a definite tryptic activity. Some years later Schepowalnikow [1899] also discovered that if intestinal juice, containing enterokinase, is added to fresh pancreatic juice, the mixture has tryptic activity, although each component of the mixture is inactive. From then onwards the two processes were generally studied independently of one another. The activation of pancreas extracts by direct addition of enterokinase was investigated in detail by Bayliss and Starling [1904] and by Mellanby and Woolley [1912]. Vernon [1901] worked mainly upon the "spontaneous activation" of pancreas extracts, although later [1914] he put forward a mechanism to explain the activation of pancreas extracts which applied both to the "spontaneous activation" and to that produced by direct addition of enterokinase. Waldschmidt-Leitz re-examined the whole question. He [1924] worked out the nature and mechanism of the activation of pancreas extracts by added enterokinase. Then, with Harteneck [1925], he investigated the problem of "spontaneous activation." They separated, from an extract which had undergone "spontaneous activation," the substance responsible for the activation. It was found that the properties of this substance were similar to those of enterokinase, and so it was concluded that "spontaneous activation" is a process identical with the activation of trypsin by added enterokinase. Further, Waldschmidt-Leitz and Harteneck by carrying out alumina adsorptions upon fresh pancreas extracts, were able to separate from the trypsin a fraction which apparently contained a precursor of enterokinase, since on keeping this fraction the activator was produced apart from the presence of trypsin. From these observations Waldschmidt-Leitz suggested that the enterokinase content of the intestinal mucous membrane depends upon the entry of pancreatic secretions in which the enterokinase is initially present in the form of an inactive pre-stage. This point of view was strengthened when Waldschmidt-Leitz and Waldschmidt-Graser [1927] showed that after removal

of the pancreas no appreciable amount of enterokinase appears in the intestinal mucous membrane even after several days.

The present paper records a number of experiments which have been carried out upon the formation of enterokinase from its pre-stage.

Experimental.

Isolation of the precursor.

This was carried out according to the method of Waldschmidt-Leitz and Harteneck, the actual procedure being as follows.

To 15 cc. of ice-cold extract (80 % glycerol) of fresh dried pancreas powder were added 15 cc. of ice-cold acetate buffer, containing 3·9 cc. *N* acetic acid, 10·5 cc. 0·2 *N* sodium acetate and 0·6 cc. distilled water. The buffered extract was then treated with 4 cc. of a suspension of alumina $C\gamma$ and the mixture left for 15 mins. in the refrigerator, when the alumina was centrifuged off. The clear liquid from the centrifuging was again treated in the same way with alumina, this process being carried out four times in all. The alumina from this series of adsorptions was then eluted with 25 cc. of 0·05 *N* ammonia. The precursor, together with peptidases, was contained in the elution which, after centrifuging off the alumina, was neutralised with *N* hydrochloric acid.

For the investigation of the development of enterokinase 5 cc. of a phosphate buffer were added to 20 cc. of neutralised elution, and the mixture contained in a glass-stoppered bottle was placed in a thermostat at 30°. A drop of toluene was always added to the mixture.

p_H *measurements.*

A sample of the buffered elution was used for the determination of its p_H value. This was determined at room temperature by means of the glass electrode. The circuit used was a modification of that employed by Harrison [1930] and involves the use of a special type of triode valve in conjunction with the glass electrode. The valve, which was supplied by Phillips Lamps Ltd., requires a filament current of 0·85 amp. and a plate voltage of 4–8 volts. A grid bias of at least -1 volt is used. The glass electrodes when not in use were kept under distilled water, and immediately before use were immersed for a few minutes in boiling distilled water. It was found that this treatment helped to ensure a low value (usually less than 10 millivolts) for the "zero reading" of a particular electrode. This is in agreement with the experience of MacInnes and Belcher [1931].

The method employed for measuring the formation of enterokinase.

The principle of the method employed was to remove a sample of the elution at various time intervals and add it to a buffered solution of trypsin at 30°. Then, after allowing a period of time for activation of the trypsin by the added sample of elution, caseinogen and buffer solutions were added to the mixture. The increase in the number of free carboxyl groups, produced by

hydrolysis of the caseinogen by the activated enzyme in a given time, was then measured. The whole procedure is carried out according to the Linderstrøm-Lang and Steenberg [1929] modification of the method evolved by Willstätter *et al.* [1926] for the determination of trypsin, and the experimental details of its application to the determination of enterokinase have previously been given by the writer [1931].

In the present case, although the principle of the method remains the same as outlined above, it has been found advisable to modify certain details of the experimental practice. These modifications have been introduced because after a preliminary series of experiments it was realised that the activation effects to be measured are of very small magnitude, as revealed by the normal method of measurement. Thus it was with the object of increasing the analytical measurements of these activation effects that the procedure was modified. At the same time it was realised that the changes should not be such as to contravene the conditions which should be observed in a method for the determination of enzyme quantity.

(1) The period of activation, 30 mins. at 30°, is kept the same as before but the total volume of the mixture during activation is increased by 1 cc., *i.e.* from 9 cc. to 10 cc.

(2) The period of hydrolysis is increased from 20 mins. to 60 mins.

(3) Titrations are carried out on 15 cc. of reaction mixture instead of on 10 cc.

The composition of the mixture used is as follows:

During activation period { 2·5 cc. enzyme solution, 3·5 cc. buffer I, 4·0 cc. elution sample;

Then there are added ... { 6·0 cc. buffer II, 15·0 cc. caseinogen solution,

thus making at the start of the hydrolysis period a total volume of 31 cc.

It is also essential in every experiment to carry out a control run in which 4·0 cc. of distilled water are used instead of the 4·0 cc. of elution, the other constituents of the mixture remaining the same. This is necessary because in the longer time now employed, 60 mins., there is measurable hydrolysis by the enzyme + buffer of itself, in all probability due for the most part to the concentration of OH^- ion provided by the alkaline buffer.

During the course of the work enzyme solutions prepared in various ways have been used. The actual preparation used in a particular set of experiments is always stated.

Results of typical experiments in connection with the method of determination are given in Table I. In these experiments the enzyme solution used was the neutralised mother-liquor remaining over from the alumina adsorptions. It therefore contained the two components of trypsin—proteinase and carboxypolypeptidase.

Table I.

Samples for activating taken from an elution, buffered with phosphate buffer at p_H 6·98, and maintained for 32 hours at 30°. Blank and control readings have been subtracted so that the increases in acidity, recorded in terms of 0·1 *N* NaOH, are a measure of the hydrolysis produced by activated enzyme.

	Increase in acidity cc. 0·1 *N* NaOH
(1) 30 mins. activation, 20 mins. hydrolysis	0·26
(2) 30 mins. ,, 60 mins. ,,	0·80
(3) 60 mins. ,, 60 mins. ,,	0·84

The following conclusions, concerned with the method of determination, may be drawn from these experiments.

(1) With the small concentrations of activated enzyme we are dealing with, it is seen that we are in the region where the hydrolysis is a linear function of time. Thus the longer period of hydrolysis, 60 mins., may legitimately be used. For obviously we are not contravening the reason for which the short period is normally employed—namely to avoid the influence of the products of reaction upon the rate.

(2) The enterokinase formed in the elution resembles enterokinase as usually obtained from intestinal mucous membrane, in that 30 mins. at 30° is adequate time for it to produce activation at its maximum. This, of course, is in agreement with the result obtained by Waldschmidt-Leitz.

It may also be pointed out, from results previously given by the writer [1931] of the determination of enterokinase that for increases in acidity up to 1·0 cc. 0·1 *N* NaOH, there is direct proportionality between such increases and the "quantity" of enterokinase responsible for them.

The rate of formation of enterokinase from the precursor.

In the first place the results of a preliminary experiment upon rate of formation are given. In this experiment the enzyme solution used to measure formation of enterokinase was a glycerol extract of dried pancreas powder. The analytical readings are set out in full, in Table II, to give some idea of their magnitude, and to illustrate a point to be made later.

It will be observed from Table II that for the zero time reading the hydrolysis produced is practically identical with that observed in the control experiment. That is, there is practically no measurable quantity of enterokinase present in the elution initially.

Table III represents the results obtained with elutions prepared separately and at different times, but in each case buffered to about the neutrality point with phosphate buffer. As will be seen later this is apparently the p_H region in which formation of activator proceeds most favourably. In Table III blank and control readings have been subtracted and the increases in acidity actually due to addition of elution are given opposite corresponding times of withdrawal. All readings have been rounded off to the nearest 0·05 cc. of 0·1 *N*

Table II.

Elution buffered with phosphate buffer at p_H 7·2, and maintained at 30°. Glycerol extract of dried pancreas used as enzyme solution in the method of determination.

The readings are given in cc. of 0·1 *N* NaOH.

Times of withdrawal of samples from buffered elution	0 hours	22 hours	46 hours
Analytical readings of total hydrolysis:			
(*a*) Titres at the end of hydrolysis period	(*a*) 21·29	(*a*) 22·16	(*a*) 22·08
(*b*) Blank titres (start of hydrolysis period)	(*b*) 20·78	(*b*) 20·80	(*b*) 20·82
$y=(a-b)$ = Total hydrolysis	(*y*) 0·51	(*y*) 1·36	(*y*) 1·26
Analytical readings of control (hydrolysis due to enzyme + buffer alone):			
(*c*) Titres at the end of hydrolysis period	(*c*) 20·77	(*c*) 20·80	(*c*) 20·81
(*d*) Blank titres (start of hydrolysis period)	(*d*) 20·30	(*d*) 20·33	(*d*) 20·35
$z=(c-d)$ = Hydrolysis due to (enzyme + buffer)	(*z*) 0·47	(*z*) 0·47	(*z*) 0·46
$x=(y-z)$ = Actual hydrolysis for which addition of elution is responsible	(*x*) 0·04	(*x*) 0·89	(*x*) 0·80

NaOH. For these experiments the enzyme solution (employed in the method of determination) was the neutralised mother-liquor from the alumina adsorptions.

Table III.

Temperature 30°

	Time of withdrawal of elution samples (hours)	Increase in acidity due to activation produced (cc. 0·1 *N* NaOH)
Elution A (p_H 7·04)	0	0·0
	10	0·50
	25	0·70
	47	0·70
	76	0·60
Elution B (p_H 6·95)	0	0·0
	7	0·45
	12	0·65
	24	0·85
	44	0·80
Elution C (p_H 6·98)	0	0·0
	10	0·45
	19	0·70
	43	0·75
	67	0·65

From Table III it is seen that, in this p_H region, maximum formation of the enterokinase is attained in about 35 hours at 30°. After this limiting value is attained there is a slow diminution, in all probability due to thermal inactivation of the enterokinase.

Although the small magnitude of the increases recorded in Table III makes it inadvisable to attach undue importance to fitting an equation to the observed results, yet the general behaviour requires some theoretical treatment. For the purposes of illustrating this treatment, an average curve from the results of Table III is given in Fig. 1.

We have two processes going on simultaneously:

(1) formation of enterokinase from the pre-stage;

(2) thermal inactivation of the enterokinase.

If we call the initial concentration of pre-stage a, and represent the amount of enterokinase produced therefrom, at any instant t, by x. Then we have:

$$\text{observed rate} = \frac{dx}{dt} = k\,(a - x) - k'x$$

where k = velocity constant of formation of enterokinase,

k' = velocity constant of thermal inactivation of enterokinase.

$$\therefore \quad \frac{dx}{k.a - (k + k')\,x} = dt.$$

On integration we have:

$$t = \frac{1}{k + k'} \log_e \frac{k.a}{k.a - (k + k')\,x}.$$

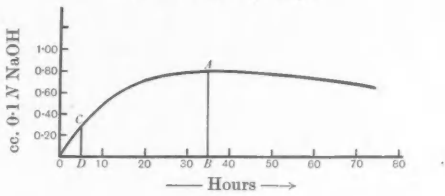

Fig. 1.

It will be observed that when k' is negligible compared with k the above equation reduces to that of the unimolecular expression:

$$t = \frac{1}{k} \log_e \frac{a}{(a - x)}.$$

Now from the original expression for the observed rate

$$\frac{dx}{dt} = k\,(a - x) - k'x$$

it is seen that when $(a - x) = 0$, the rate is given by $-k'x$. So that if we assume that the diminution from the limiting value at 35 hours is due to thermal destruction of the enterokinase, we can calculate from this rate of diminution a value for k'. Taking, from the curve, values of 0·80 and 0·68 as representing the quantity of enterokinase at 35 hours and at 70 hours respectively, then with t in mins. the value of k' works out to be $7{\cdot}9 \times 10^{-5}$. We may now check our assumption, since we can also calculate a value for k' from previous data of the writer [1931] upon the thermal inactivation of enterokinase. From those data we have

(1) between p_{H} 6·0 and p_{H} 7·0 the average $k_{\text{inactivation}}$ at 50° is $4{\cdot}42 \times 10^{-3}$, when t is in mins.;

(2) the observed critical increment, E, for the inactivation reaction is about 40,000 calories.

$\therefore$ substituting in the equation

$$E = \frac{2{\cdot}303\,RT_1T_2}{(T_1 - T_2)} \log_{10} \frac{k_{T_1}}{k_{T_2}}$$

we have

$$\log_{10} \frac{4{\cdot}42 \times 10^{-3}}{k'} = \frac{40{,}000 \times 20}{2{\cdot}303 \times 1{\cdot}99 \times 323 \times 303},$$

whence $k' = 7{\cdot}3 \times 10^{-5}$.

This value shows agreement with that calculated on the basis given previously. Therefore we may now examine how a value for k' of this order affects the observed rate of formation. This may be done in the following way. We may calculate the percentage diminution occurring in 15 hours from a value for k' of this order. Thus we have, putting t in mins.,

$$7{\cdot}6 \times 10^{-5} = \frac{2{\cdot}303}{900} \log_{10} \frac{a}{(a-x)},$$

whence $\left(\frac{a}{a-x}\right) = 1{\cdot}07$. If we put $a = 1$, then it is seen that $(a - x) = 0{\cdot}94$. That is the percentage diminution in 15 hours is about 6 %. It will be seen from Fig. 1 that after 15 hours the quantity of enterokinase present is represented by 0·64 cc. An error of 6 % in 0·64 cc. means a change of 0·04 cc. Over the period from 15 hours to 30 hours the increase in quantity of enterokinase present is $(0{\cdot}80 - 0{\cdot}64) = 0{\cdot}16$. The correction on this value is 0·01 cc. Corrections of this order are no greater than the experimental error. This means then, in effect, that we may neglect k' in comparison with k, and that the limiting quantity of enterokinase formed (AB in Fig. 1) may be taken without serious error to represent a, the initial concentration of precursor. So that if, as we assumed originally, the true rate of formation of the enterokinase is a function of "active mass" of precursor alone, then, since we may neglect k', the observed rate should agree with that predicted by the unimolecular expression.

This comparison has been made by taking as the value for a the maximum quantity of enterokinase formed (AB in Fig. 1) and taking one value of x from the curve (distance CD). From these k is worked out and found to be, t in mins., $1{\cdot}57 \times 10^{-3}$. This value of k is then used to calculate x for different time intervals, and these values are then compared with those observed. This is shown in Table IV, where calculated values for a bimolecular constant $k_{bi} = \frac{1}{t} \cdot \frac{x}{a(a-x)}$ are also given.

Table IV.

Time (hours)	Increases in acidity: Calculated from k_{uni}	Observed	Calculated from k_{bi}
7	0·39	0·38	0·36
10	0·49	0·50	0·44
15	0·61	0·62	0·51
20	0·68	0·72	0·56
25	0·73	0·76	0·60

From Table IV it is seen that the rate of formation is apparently best represented by the unimolecular expression.

The effect of the p_H *of the elution upon the formation of enterokinase.*

The effect of p_H was studied in the following way. A volume of 60 cc. of neutralised elution was prepared from 60 cc. glycerol extract in the manner already described. Then 2 cc. of various buffer solutions were measured out into

each of a number of glass bottles. To each of these a volume of 8 cc. of the neutralised elution was then added, making a total volume of 10 cc. in each bottle. In every case a sample of the mixture was withdrawn for the determination of the "zero reading," and a sample for p_H determination. The bottles were then stoppered and kept in a thermostat at 30° for 16 hours. At the end of this period the quantity of activator formed in each case was measured in the way already described. Results obtained in this way are given in Table V.

Table V.

Temperature 30°. Time 16 hours.

	Buffer used	p_H	Increase in acidity cc. 0·1 *N* NaOH
Elution X.	Acetate	5·0	0·45
	Phosphate	5·95	0·74
	,,	6·57	0·85
	,,	6·95	0·89
	,,	7·20	0·68
	Ammonia-ammonium chloride	7·90	0·43
Elution Y.	Phosphate	5·60	0·41
	,,	6·35	0·70
	,,	6·92	0·76
	,,	7·20	0·70
	,,	7·40	0·64
	Ammonia-ammonium chloride	8·00	0·35

Blank and control readings have been subtracted. The enzyme solution used (in the method of determination) was new neutralised mother-liquor from alumina adsorptions. With the phosphate buffers the total phosphate in the mixture in each case was 0·016 *M*.

When the results given in Table V are plotted it is found that the same shape of curve is given by both elutions. The optimum region for formation of the enterokinase appears to be about p_H 6·5–7·0. The rate drops off quite sharply on the alkaline side, less so on the acid side. It now remains to examine whether the p_H effect observed is truly an effect upon formation or whether the observed effects can be explained on the grounds of increased (thermal) inactivation away from the neutrality point. This may be done by again referring to data upon the inactivation of enterokinase. We have already shown that the inactivation effect may be neglected around neutrality, and as the following values (taken from the writer's previous data) show, there is very little increase in the rate of inactivation from neutrality to p_H 5 on the one side, and to p_H 8 on the other. It is beyond these limits that the thermal inactivation becomes very markedly increased. The values now given refer to a temperature of 55°.

p_H	$k_{\text{inactivation}} \times 10^2$
5·12	1·30
6·40	1·15
7·83	1·30

It is therefore seen, by comparison of these data with those of Table V, that over approximately the same p_H range the rate of inactivation of

enterokinase only increases by about 10 % from the optimum, while the rate of formation of enterokinase decreases by about 50 % from the optimum.

Thus from these figures it may be concluded that the p_H effect observed arises in fact from the influence of p_H upon the rate of formation of the enterokinase.

Further experimental work is in progress, partly with the object of investigating whether the peptidases, present in the elution with the precursor, are concerned in the formation of the enterokinase therefrom.

Summary.

(1) The separation, from pancreas extracts, of a pre-stage of enterokinase (as recorded by Waldschmidt-Leitz and Harteneck) has been confirmed.

(2) Experimental details of a modified method for measuring the formation of enterokinase from this pre-stage are given.

(3) It is found that the enterokinase produced in this way resembles enterokinase as it is usually obtained from the intestinal mucous membrane in that 30 mins. at 30° is adequate time for it to produce maximum activation of trypsin. This is in agreement with the experience of Waldschmidt-Leitz and Harteneck.

(4) It is found that the enterokinase produced is able to activate the trypsin in both purified and unpurified extracts.

(5) The rate of formation of the enterokinase at 30° has been measured, and some theoretical considerations upon the rate of formation are given. In the course of these it is shown that the rate of formation may be expected to agree with the unimolecular expression. The observed rate is compared with that predicted (*a*) by the unimolecular expression and (*b*) by the bimolecular expression. It is found that the observed rate agrees better with (*a*) than with (*b*).

(6) The effect of p_H upon the rate of formation has been observed. It is found that the optimum p_H of formation is about p_H 6·5–7·0. It is also shown that this observed effect is not due to increased thermal inactivation at points away from this region, but represents essentially the effect of p_H upon the formation of enterokinase.

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LXXXII. THE CHEMISTRY OF COOKING.
II. ON THE CRITICAL TEMPERATURE IN COOKING THE SWEET POTATO.

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In the preceding article on this subject [Sinoda, Kodera and Oya, 1931] it was shown that polymerisation of soluble sugars and hydrolysis of polysaccharides occur synchronously when the sweet potato is treated with water at 100°. In cooking the sweet potato, it is not necessary to heat the material at so high a temperature as 100°, and this paper is concerned with finding out the lowest temperature at which the sweet potato may be cooked, and also to confirm the chemical changes of the carbohydrates, which occur in the process of cooking.

I. Morphological change in cooking.

When cooked, not only does the sweet potato increase in sweetness, but its tissue becomes considerably softer; these changes in consistency are accompanied by a change in the morphology of the tissue. Sections of thickness from 150 to 200 μ were prepared by Schanze's microtome from the cooked and uncooked pieces without fixation or imbedding, and stained with dilute iodine-potassium iodide solution. Without dehydration, the section was mounted in a modified Berlese's solution of the composition: chloral hydrate 160, gum arabic 15, cane sugar 20, and water 25; the solution gave a good result in taking photomicrographs.

Starch granules in the fresh material bunch together in the middle of the cells, and stain bluish-violet with the iodine solution, while the granules in the cooked material, losing their normal globular form, swell, gelatinise and coalesce, so that they fill almost the whole cavity of each cell (Fig. 1). Moreover, the starch granules of the cooked potato can be distinguished from the fresh ones by the fact that they give a transient pure blue colour with the iodine solution.

The gelatinisation temperature of the starch of the sweet potato will therefore have an intimate connection with the critical temperature for cooking. The former is determined experimentally to be 74°, and is the same as that of the American sweet potato starch [Reichert, 1913].

In the experiment, a cylindrical piece of sweet potato of diameter about 3 cm., which was prepared by cutting along its axis, was dipped into hot water

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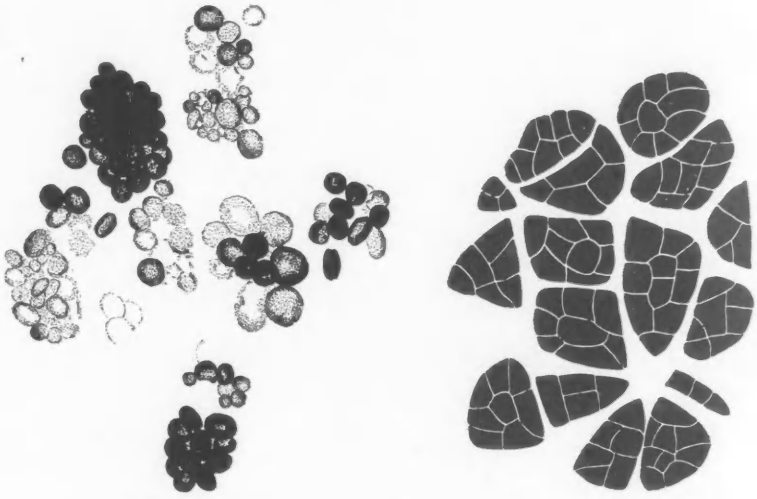
at the desired temperature, and the change of the temperature was measured by a thermometer inserted into the middle of the piece.

Experimental results indicated that the sweet potato became agreeably edible when it was kept over 80°, but not when kept at 50°, 60°, or 70° for



a *b*

Fig. 1. Starch granules before and after cooking. *a*, fresh; *b*, steamed.

more than 30 minutes, and the microscopic appearance of the starch granules from the potatoes kept under 70° was similar to that observed in the fresh piece.

Thus, the critical temperature for cooking the sweet potato was determined to lie between 70° and 80°, and coincides with the gelatinisation temperature of its starch.

II. Chemical changes in cooking.

A. *Material and method.*

The sweet potato used for these experiments had the composition shown in Table I.

Table I. *Composition of the raw material.*

Exp. No.	I	II	III	IV	V	VI	VII	VIII	IX	X
Locality	Terada, Kyoto-fu	Mukojima, Kyoto-fu	Saruta, Chiba-ken	Saruta, Chiba-ken	Terada, Kyoto-fu	Koma, Kyoto-fu	Nagao, Osaka-fu	Terada, Kyoto-fu	Terada, Kyoto-fu	Terada, Kyoto-fu
Date of exp.	13. xi	27. x	12. vi	9. vi	30. x	2. vi	17. xi	20. xi	8. xii	14. xii
Moisture	64	62	65	59	64	60	57	63	62	63
Monosaccharides	1·7	2·1	12·2	11·9	—	15·0	2·1	2·4	4·0	4·4
Disaccharides	5·0	3·4	15·6	14·8	—	21·3	2·8	4·0	5·3	8·2
Polysaccharides (dextrins)	7·4	2·6	11·1	29·8	—	45·8	1·8	4·9	3·7	3·1
Starch	55·9	53·2	28·7	32·4	—	15·8	77·4	72·2	63·1	44·8
Sp. Gr.	—	—	—	—	—	—	—	—	1·07	1·07

Steaming at constant temperature. A boat containing the sample was introduced into the inner jacket of an Abderhalden pistol in which were also placed two pieces of cotton-wool soaked in water. The drying-bulb of the apparatus was replaced by a cork carrying a thermometer.

Stewing at constant temperature. A similar method was used in Exps. III, IV and V. In other cases several pieces of a certain diameter (say 4·5 cm.), cut from a large tuber, were at once dipped in a hot water pan which was

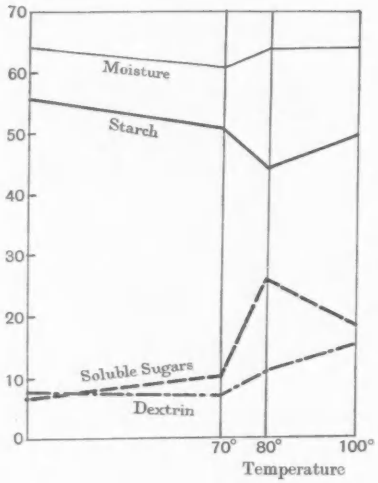


Fig. 2. Stewing for 20 minutes (I).

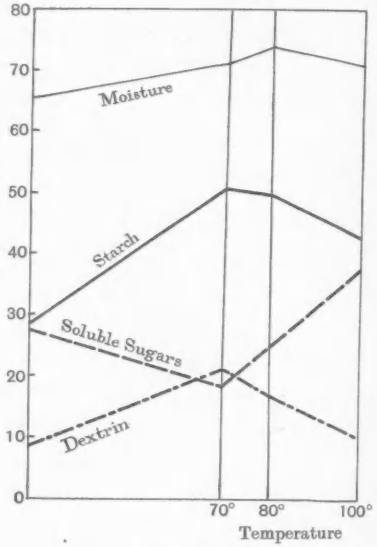


Fig. 3. Stewing for 1 hour (III).

heated with a burner to reach the desired temperature within 5 minutes, the temperature being measured by a thermometer inserted into the centre of one of the blocks. When cooked the outer part of the block was removed with a knife and the inner portion was used for analysis.

Moisture was determined by distilling with toluene in Kafuku's apparatus (Jap. Pat. 63210).

Reducing sugar was determined by Willstätter's iodimetric method, using about 20 g. of the sample which was ground to a paste before the experiment. In Exps. III, IV and V, the part soluble in hot water was separated from the insoluble residue and analysed as reported in the preceding paper [Sinoda *et al.*, 1931]. In all other experiments, 20 g. of the sample were taken in a flask and made up to one litre. 100 cc. of the mixture were centrifuged to separate the precipitate which was well washed with water and hydrolysed with HCl to

estimate the starch content. The clear solution separated completely from starch suspension was used for the estimation of soluble sugars and dextrin by the usual method, and the results are shown in Tables I–IV.

B. *Stewing.*

(*a*) *Stewing for a short time.* When the sweet potato is stewed at 70°, 80°, or 100° for 20 minutes (Exps. I and II) the starch content shows a slight decrease at 70°, which is more marked at 80°, but less again at 100°. The content of soluble

Table II. *Chemical changes in 20 minutes' stewing.*

Exp. No.	Temperature	Moisture %	% of dry weight as Monosacch.	Disacch.	Polysacch. (dextrin)	Starch
I	Unheated	64	1·7	5·0	7·4	55·9*
	70°	61	4·9	5·2	7·1	50·7
	80°	64	22·7	3·4	11·0	44·5
	100°	64	14·3	4·5	15·7	49·6
II	Unheated	62	2·1	3·4	2·6	53·2
	70°	66	12·5	8·5	9·3	49·6
	80°	61	20·0	9·1	14·0	41·7
	100°	63	11·7	5·6	8·0	43·2

* The sum of total carbohydrates is not always the same in the cooked and fresh materials. This perhaps depends upon the method of calculation (*cf.* the former paper), and a further study is necessary to settle the point.

sugar changes inversely with that of starch; in other words starch is partly hydrolysed between 70° and 80° (the gelatinisation point is 74°) while soluble sugars polymerise at higher temperatures.

(*b*) *Stewing for longer times.* When the samples (of chemical composition different from those used in Exps. I and II) were stewed for 1 and 2 hours respectively (Exps. III and IV), some hydrolysis was observed at 70°–80°, and reduction of the starch by this means will continue even at higher temperatures such as 100°. During prolonged stewing however some increase in the content of the starch fraction was to be seen at 70° due to the polymerisation of soluble polysaccharides, a change which was not observed in Exps. I and II.

Table III. *Chemical changes in prolonged stewing.*

Exp. No.	Duration of stewing	Tempera-ture	Moisture %	% of dry weight as Mono-sacch.	Disacch.	Polysacch. (dextrin)	Starch
III	1 hour	Unheated	65	12·2	15·6	11·1	28·7
		70°	71	9·7	8·9	21·3	50·9
		80°	74	13·0	11·7	17·1	49·8
		100°	71	22·0	15·0	10·3	42·5
IV	2 hours	Unheated	59	11·9	14·8	29·8	32·4
		70°	62	12·1	10·3	31·1	47·9
		80°	68	15·6	10·0	19·8	45·0
		100°	69	19·2	11·5	19·1	45·9

(c) *Analysis together with cooking water.* The material was stewed at 70°, 80° and 100° respectively for 1 hour and was analysed together with the water used for cooking (Exp. V). As indicated in Table IV, a considerable decrease of the soluble sugar fraction was noticed.

Table IV. *Chemical changes by stewing (water for cooking included in the analysis).*

Exp. No.	Duration of stewing	Temperature	Moisture %	% of fresh weight as Monosacch.	Disacch.	Polysacch. (dextrin)	Starch
V	20 mins.	Unheated	64	—	—	—	—
		70°	—	8·9	4·4	9·4	11·5
		80°	—	8·3	7·3	15·9	4·0
		100°	—	7·7	2·6	17·2	4·2

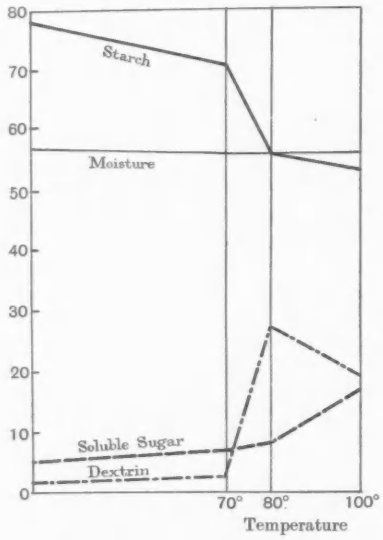


Fig. 4. Steaming for 2 hours (VII).

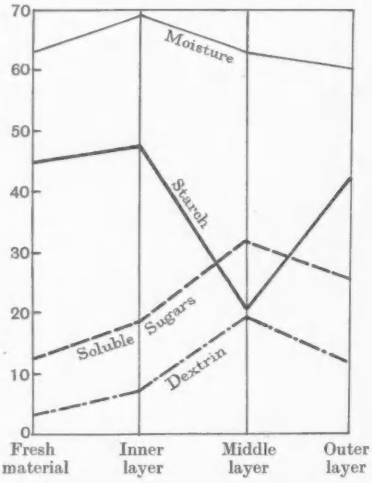


Fig. 5. Incomplete stewing (X).

C. *Steaming.*

A sample of lower starch content steamed for 2 hours at various temperatures (70°, 80° and 100°) (Exp. VI) underwent chemical changes as regards the starch fraction similar to those observed during brief stewing (Table II), while the samples with higher starch contents (Exps. VII and VIII) underwent hydrolysis as the principal reaction, resembling in this the samples subjected to prolonged stewing (Table III).

Table V. *Chemical changes during steaming for 2 hours at various temperatures.*

Exp. No.	Temperature	Moisture %	% of dry weight as Monosacch.	Disacch.	Polysacch. (dextrin)	Starch
VI	Uncooked	60	15·0	21·3	45·8	15·8
	70°	—	14·5	10·3	45·5	26·0
	80°	—	22·8	21·5	28·4	15·3
	100°	—	19·8	10·8	31·8	33·3
VII	Uncooked	57	2·1	2·8	1·8	77·4
	70°	56	3·4	3·0	2·5	70·7
	80°	56	8·2	0·02	27·7	55·7
	100°	56	14·9	1·8	18·5	53·0
VIII	Uncooked	63	2·4	4·0	4·9	72·2
	70°	59	3·4	4·9	4·4	57·2
	80°	58	17·0	7·4	—	46·8
	100°	57	16·0	7·9	14·4	43·7

D. *Incomplete stewing.*

(*a*) *Method.* As mentioned above, we noticed that, in general, hydrolysis of starch occurs at 70–80°, and polymerisation of soluble sugars at higher temperatures. However, the chemical changes in the sugars during cooking (stewing or steaming) were also observed to differ in some degree according to the content of starch and also to the treatment. For the further elucidation of these points the following experiment was carried out. A large cylindrical piece of sweet potato (diameter 4 cm.) was taken, and a thermometer was inserted into its centre. The piece was plunged in boiling water, left until the thermometer registered 50°, and then removed and allowed to cool (during cooling the thermometer rose about 10°); the periphery of the piece was well-cooked, the centre remained quite raw and the middle layer was half-cooked.

(*b*) *Morphological examination.* A transverse section of the above-mentioned block, stained with dilute iodine solution, shows under the microscope the following three layers.

(i) Inner layer: Starch granules separate, globular, bunched together, and stained blue-violet, and similar in general appearance to those of the fresh material (Fig. 1).

(ii) Middle layer: Starch granules gelatinous, swollen, and coalesced; stained blue like those of the cooked material (Fig. 1).

(iii) Outer layer: Starch granules similar to those of the middle layer, but iodine coloration transient. The decoloration of the starch granules seems to be due to the reduction of iodine. Thus, when the section was soaked with neutral silver nitrate solution, the layer blackened in a few moments.

(*c*) *Chemical analysis.* The above three layers were carefully cut out under microscopic control and were separately analysed, the results of two such experiments being shown in Table VI. As is to be expected, the outer layer is quite rich in the starch fraction while the middle layer is rich in di- and poly-saccharides. These facts indicate clearly that the polymerisation of

Table VI. *Chemical changes in incomplete stewing.*

Exp. No.	Position of sample	Duration of boiling	Moisture %	% of dry weight as Monosacch.	Disacch.	Polysacch. (dextrin)	Starch
IX	Untreated	15·0 mins.	62	4·0	5·3	3·7	63·1
	Inner layer		59	4·6	4·8	3·1	55·8
	Middle layer		54	11·1	5·2	11·5	42·4
	Outer layer		62	14·1	3·4	10·9	43·5
X	Untreated	13·5 mins.	63	4·4	8·2	3·1	44·8
	Inner layer		69	4·9	13·7	7·2	47·6
	Middle layer		63	19·6	12·3	19·8	20·7
	Outer layer		60	14·7	10·9	11·4	42·2

soluble sugars predominates in the former part and hydrolysis in the latter. The chemical changes which occur in the carbohydrates of the inner layer are to be attributed to the effect of temperature and especially to pressure.

E. *Change of* p_H.

p_H was determined colorimetrically. A test-paper was placed between two blocks (fresh or cooked), pressed for a definite time, and the colour of the paper was compared with the standard. The experimental results (average of three determinations) indicate that the p_H changes from 6·2 to 5·7 during stewing (the sample was the same as used in Exp. X).

Discussion.

In Figs. 2–5 some of the above results are shown graphically.

Physiological, morphological and chemical studies show that the sweet potato when cooked at 70°–80° becomes agreeably edible and by this treatment the starch of the sweet potato undergoes partial hydrolysis. At higher temperatures than 80° the formation of polysaccharides seems to begin at the cost of soluble sugars, although the latter phenomenon may be masked by continued hydrolysis (*a*) when the starch content of the sample is too high, (*b*) when the moisture content is too low, (*c*) or when the cooking is prolonged.

In the case of steaming the polymerisation of simple sugars takes place in some degree even at a lower temperature, such as 70°. The increase in the content of the starch fraction by cooking may result partly from the loss of soluble sugars by solution in the cooking water.

However we cannot deny the actual formation of polysaccharides when we refer to Exp. V in which a considerable decrease in the content of soluble sugar was noticed even when the content of sugar in the cooking water was allowed for.

The fact that, as was reported in the preceding paper [1931], the digestibility of the potato starch by diastase is markedly increased by cooking, indicates that the chemical nature of the polymerides formed during cooking is not entirely similar to that of ordinary starch.

The taste of the potato is evidently improved by cooking, and the process of cooking and time of heating are also considered as important factors for improving the taste and the digestibility. As a matter of fact steaming gave a better taste than stewing; similarly stewing at 100° gave a better result than at 80° and stewing for 1–2 hours at 80° was more advantageous than the same treatment for 20 minutes at this temperature, owing to increase of the content of new polymerised sugars of unknown nature. In practice, we can recommend steaming the sweet potato at 100° in order to get a good food material, unless the potato has a high content of starch or low content of water, when boiling with plenty of water is preferable.

In the present experiment, we noticed that the fresh materials differed in chemical composition from those of the former experiments [1931], owing to the difference of the seasons in the sampling. In the previous work stored sweet potatoes were used as samples, and consequently the starch was partly converted into soluble sugars during the preservation, whereas in the present experiment very fresh potatoes were used having a high starch content.

Summary.

1. The sweet potato becomes edible with improved flavour when it is stewed or steamed at temperatures higher than the gelatinisation point of its starch (74°).

2. A remarkable morphological change in the starch granules is observed when the sweet potato is cooked.

3. Partial hydrolysis of starch in the tissue occurs between 70° and 80°.

4. At temperatures higher than 80°, polymerisation of lower sugars begins.

5. The taste is largely influenced by the content of these unknown polymerisation products.

6. In cooking, it is generally advisable to treat the sweet potato at higher temperatures for shorter times.

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LXXXIII. ESSENTIAL OILS AND HEAT ABSORPTION.

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Efficiens est calor Solis qualitatem educens et extrahens. (*Themata physica de odorum natura et affectionibus.*) [Iohannes Camerarius: Marburg; 1587. Quoted by Kenneth, 1928]. Tyndall [1875 and earlier] was the first to establish that water vapour in very small amount appreciably absorbed heat in the form of radiant energy. His finding was confirmed spectroscopically by a large number of workers and is now a scientific commonplace. Tyndall also investigated the heat absorption of essential oil vapours, but, although his work is mentioned by many botanists and writers on perfumes, only one later worker appears to have investigated this question experimentally. Grijns [1919], who used a modification of Tyndall's apparatus, included only three essential oils in his series; but he found absorption of heat by volatile substances to be much smaller than the degree claimed by Tyndall.

A quantitative estimate of heat absorption by vapour is of possible importance to the evaluation of essential oils, and in problems of the physiology of odour, and of the rôle of the essential oil in the plant. An attempt was made to investigate the heat absorption of odoriferous substances. Two methods were used. The first was based upon the conversion of intermittent energy into sound [Tyndall, 1880, 1]. It was hoped that this method might be made sensitive by the use of thermionic amplification, but it was not possible to find reliable conditions or to obtain sufficiently intense sounds. Tyndall [1881, 2] made some remarkable claims as to the intensity and variation of sound obtainable but the experimental details he gave are meagre. It is perhaps noteworthy that Rayleigh [1903], in discussing this part of Tyndall's work, made no reference to any intensity of sound greater than that obtained by the present author. This part of the work was abandoned in favour of the thermopile method.

Preliminary experiments with vapours in a long glass tube through which radiant heat was sent to impinge on the face of a thermopile in air, failed to show any appreciable absorption due to the vapour of essential oils. At an early stage the difficulty of obtaining sufficiently large plates of rock-salt became evident. Several suggested substitutes were tried and found to be nearly opaque to radiant heat originating at the temperature of boiling water.

A special journey to the region of Salzburg, in Austria, and to Stassfurt, in Germany, was made [Nicol, 1928] and a supply of salt obtained. Much time was spent in acquiring the technique of working suitable pieces into plates.

Preliminary experiments, in which the vapour was enclosed between rock-salt plates in a glass tube, were considered inconclusive owing to the double passage of the heat rays through air at entry into and exit from the tube. Tyndall took elaborate precautions to pass his entering rays through a vacuum only, but he placed the receiving thermopile in air. Although Tyndall's thermopile was "compensated," it appeared desirable to make the apparatus self-contained. The final form, which was intended to be tentative only, was as follows.

A vertical brass tube 5·3 cm. internal diameter and 53 cm. long was closed at the upper end by a brass chamber through which steam was passed. The common wall of this chamber thus supplied the source of heat at approximately 100°. At the lower end was a removable circular diaphragm supporting a thin plate of rock-salt. Beneath this was a chamber forming a prolongation of the tube, and containing a thermopile cube insulated from the bottom of the tube. The lower end of the brass tube was immersed in a bath in solid paraffin wax to keep the temperature of the thermopile chamber approximately constant. Leads to a galvanometer were provided. An inlet tube for vapour was provided just below the steam-chest. In series with this inlet was a wider glass tube into which a porcelain boat or other container for essential oil could be inserted; during part of the experiment this tube was fitted with leads to a gold-leaf electroscope from wires dipping into the oil. Before passing over the oil-boat, air was freed from carbon dioxide and moisture by means of an apparatus consisting of 10 tubes each a metre long filled, in order, respectively: 2 with 40 % caustic soda solution, 2 with soda lime, 2 with concentrated sulphuric acid, 1 with "20 %" fuming sulphuric acid and 3 with sulphuric acid. An outlet was provided just above the diaphragm supporting the salt plate, and an exhaust tube was also provided for the thermopile chamber. The two latter tubes were connected, and stopcocks were fitted so that either or both chambers could be put into communication with each other or with a vacuum pump.

The apparatus in principle resembled that of Magnus [1861] more than Tyndall's and was open to the objection that it was impossible with it to eliminate entirely the effects of convected as distinct from radiant heat. It was also crude in that it failed to exclude heat conducted along or radiated from the walls of the tube, and that the thermopile was not compensated. It would now be possible to design a more satisfactory apparatus, although this would be extremely costly.

The design of the apparatus was not, however, of importance. Work performed with it suggested—as will be shown—that frothing of the oil indicated the presence of water in practically all of the substances tested, and this phenomenon of frothing took place outside, and was independent of, the thermostat chamber. On account of this simple demonstration of a factor of which the importance had been underestimated by Tyndall and ignored by later commentators, it became superfluous to refine the design of the apparatus, inasmuch as it was intended to detect the absorption of radiant heat by commercial samples of essential oils.

As a preliminary *experimentum crucis*, many attempts were made to determine whether the heat absorption from a source at approximately 100° of dry air was negligible, as found by Tyndall. When the rock-salt plate was not in position, and the air was allowed to come into contact with the thermopile

(the galvanometer reading having become steady with the tube exhausted or nearly so) a reading was in all cases obtained, which indicated heat absorption. Only very little less absorption was indicated when dry air was used than when room air was used. The reading with dry carbon dioxide was similar to that with dry air. With dry hydrogen, an enormous "absorption" was indicated in similar circumstances, but the absence of any indication of heat absorption by hydrogen when that gas was prevented by the salt plate from surrounding the thermopile cube, suggested that the apparent absorption was due to the combined cooling effects of conduction and convection upon the thermopile itself[1]. It was therefore decided to leave the question of the absorption by water vapour and investigate the problem of the absorption by volatile essential oils.

The first experiments with oils were, as before, made while allowing air to come into contact with the thermopile, before entering the exhausted tube, but the air was passed over the essential oil. Alternatively, the chamber containing the vessel of essential oil was exhausted either simultaneously with the tube, or by putting the oil chamber into communication with the tube after the latter had been exhausted. The last mentioned process was sometimes repeated in order to reach a sufficient degree of exhaustion in the apparatus. It was thus hoped to obtain indications of absorption (*a*) of the oil vapour in presence of varying pressures of air, and (*b*) of the oil vapour in equilibrium with oil, which was in all cases at room temperature.

In the course of these experiments, however performed, it was noticed that the oil appeared to froth or "boil" when the pressure became sufficiently low. Moreover, this phenomenon having occurred once with a given sample of oil, could not usually be repeated with the same sample of oil, however high the vacuum might be or however long it might be maintained. If the pressure were reduced in stages as mentioned above, it sometimes happened that a small degree of frothing was followed by one more intense at a lower pressure, after which the frothing could no longer be induced even with a high degree of vacuum. Simultaneously with the frothing, a deflection of the galvanometer was noticed, indicating apparent absorption of heat, but when the frothing had ceased the galvanometer reading returned to its former level. These findings were repeatedly confirmed with various oils. In one case (after the frothing) several cc. of geranium oil distilled over at room temperature and condensed beyond the experimental tube, in the tubing leading to the pump, without any deflection of the galvanometer being seen during the passage of so much supposedly heat-absorbing material through the tube.

It was concluded that the frothing, and the absorption of the heat which accompanied it, were due to the evaporation of water from the oil. Chemical consideration of the comparative water-solubilities of the constituent oils gave support to this hypothesis. A qualitative study was made of the electrical

[1] The well-known experiment of the glowing wire successively *in vacuo* and surrounded by hydrogen offers an analogy.

conductivity of the essential oil used, with a view to seeing whether any relation existed between the conductivity and the amount of frothing exhibited by the oils under the conditions described. For this purpose the device mentioned above was used, but the gold-leaf electroscope was found to be too sensitive an indicator; its reading was nearly as large in most cases after the frothing of oils as with the original oil. A modified method was devised of determining the order of conductivity and an account of results obtained with it has been published [Nicol, 1929]. It was found that the intensity of frothing of the oils, and their ability to conduct electricity, were very closely related.

With the salt plate in the tube, further experiments were made upon essential oils, and the frothing effect was disregarded. It was found that a steady current of dry air passed over oil from which the water had been almost completely boiled off (as determined by the cessation of frothing) had an almost insignificant heat absorption, hardly exceeding the experimental error. The magnitude of the effect varied and it was apparently zero with some oils.

Pure esters and other organic compounds were examined in addition to essential oils. The four benzoates tried showed diminishing water content with increasing molecular weight. The following is a list of substances examined, named in the order of increasing degree of frothing under diminished pressure. In some cases samples from two sources were tested. The order closely follows the order of the electrical conductivities, and turbidities on addition of turpentine [Nicol, 1929]. The groups are necessarily not sharply distinguished.

I. Hydrophobe group.

(*a*) Turpentine.

(*b*) Benzyl benzoate: *iso*amyl benzoate: safrol; oils of rosemary, white thyme[1], red thyme, aniseed (badiane), bergamot, limes distilled, eucalyptus (globulus), lemon, sweet (Portugal) and bitter orange, limes expressed[2], lavender (French), cananga, mandarine (tangerine), ylang-ylang, cajuput, citronella (Ceylon), patchouli.

II. Hydrophile group.

Oils of citronella (Java), petitgrain, cinnamon bark; ethyl benzoate, methyl salicylate, methyl benzoate; oils of palmarosa, cloves (English distilled), geranium (African), cinnamon leaf, lemon-grass; anisaldehyde; oil of cassia.

Discussion.

No worker familiar with Tyndall's researches on the absorption of heat by water vapour could avoid being impressed by the extreme care which was taken by Tyndall in most of his work to remove minute traces of water vapour from the dried gases which he used. With the chemistry of the essential oils,

[1] Redistilled oil of red thyme. [2] And subsequently distilled.

Tyndall appears to have had no especial acquaintance. He was aware that they might contain small amounts of water, and it is noteworthy that in his experiments on the absorption of heat by essential oil vapours, Tyndall practically neglected the possibility of an effect due to water vapour. He remarked that the amount of water vapour could only be "infinitesimal." It is also striking that perfumery workers who have subsequently commented on this work, have, presumably through ignorance of Tyndall's complementary investigations, failed to realise the importance of the water content of most essential oils. The amounts of water present in commercial oils are, in general, quite appreciable. The present author, like others conversant with the chemistry of essential oils, also overlooked the possibility of interference from water vapour until the point was brought home to him by the "frothing" mentioned above, for which no explanation suggested itself immediately.

The initial conductivity of the oils ran apparently *pari passu* with the intensity of the "frothing" they exhibited under reduced pressure. No figures are available in support of this point, since the "frothing" was not susceptible of measurement.

It was found that the least frothing and the least conductivity were possessed by turpentine, safrol, benzyl benzoate, and the oils of the citrus fruits. The greatest frothing and the maximum of conductivity were shown by lemon-grass oil, cinnamon leaf oil, cassia oil and anisaldehyde. It appeared, generally speaking, that the water contents of the oils varied inversely with their terpene contents and directly with their phenol and aldehyde contents. A confirmation of the two foregoing methods of ranging the oils in their order of water content was afforded by the qualitative examination of the turbidity produced when each was added to turpentine.

The botanical significance of these results is quite different from that which it was sought to determine. It was originally desired, *inter alia*, to test the hypothesis that some essential oils acted as a heat screen for the plant which elaborated them. This hypothesis could not be of general application to all oils, since there are some, such as camphor, which normally do not come into contact with the surrounding air, and others, such as the citrus oils which are elaborated in proximity to the exterior walls of a part of the plant yet do not appear to volatilise appreciably from it.

Dixon [1914] wrote: "It is a matter of frequent observation that many plants which are natives of arid regions secrete a relatively large amount of ethereal oils. It has been urged that the vapours of these ethereal oils form a screen which arrests the heat radiations, and thus the leaves of the plant are kept cooler than otherwise would be....Such an absorptive screen in contact with the leaves...would rather tend to raise their temperature." He suggested that the action of essential oil vapour in checking evaporation and transpiration would afford a simpler explanation of the function of the oily secretions. In a simple experiment he found that vapour given off from chopped leaves of *Artemisia absinthium* considerably reduced the rate of

transpiration of a branch of *Syringa vulgaris*. Dixon's researches might possibly be extended with advantage.

The outstanding botanical outcome of the present results is the distinction, by a method independent of any hypothesis concerning the structure or habit of the plant, of oils borne by plants of xerophyte type (conifer, labiate (rosemary and thyme) and citrus fruits), and those oils elaborated in continuous intimate contact with aqueous liquids. In the first class, the oil is formed during a desiccative process, and is hydrophobe; in the second class, the oils are continuously formed in and remain in contact with, the protoplasm until they are volatilised. It is possible that oils of the second class are frequently present as glucosides [*cf.* Hampton, 1925].

It would be of interest to compare, from the standpoint of water-retaining capacity, oils from flower and fruit of the same plant, as, for example, the orange, which yields orange-flower oil, petitgrain oil (from immature fruits) and orange oil from the *zeste*. Owing to the cost of the flower oil, and to the practical impossibility of obtaining a sample of indubitable origin, this point has not been tested as regards orange-flower oil, but it was found that petitgrain oil had indications of an appreciably higher water content than ordinary orange oil from peel of the mature fruit. In amplification of this finding, it may be noted that orange-flower water, like rose water (which contains notable amounts of the water-soluble alcohols) is an article of commerce, whereas the water separated from orange oils is of no commercial value and contains only traces of odoriferous substances.

With regard to the heat-screening hypothesis, it is evident that this hypothesis can apply if at all, only to some of the odoriferous oils secreted by plants. Whether it applies to any of them appears doubtful, and the author shares the view of Dixon concerning the physical dubiety of the hypothesis. Schübeler [1880] found in "vegetation developed under the influence of short summers with almost continuous light" "that the aroma of fruits is increased and that the development of essential oils in certain plants is greater than in the same plants grown in other latitudes."

Freund [1904] quotes a statement that the effect of coloured light on growing plants "is greatest on the scent. Thus, strawberries grown under red glass have a wonderful aroma, and crassula flowers, which are nearly scentless in ordinary sunlight, emitted a delicious fragrance, like that of bananas, under the influence of red light."

Commercial essential oils are usually steam-distilled, and contain water derived from the distillation. It was such oils, not further dried, which were used by Tyndall, and by the author in the present work. Do these commercial oils contain water in amount different from that which they contain in the plant? What are the phases of the oil and the water?

In view of the difficulty that exists in determining the absolute water content of commercial oils, the first question appears likely to remain open. The second question is of greater interest. In the estimation of relative water

content by a conductivity method it was assumed that the ordinary oil is either (*a*) a suspension of oil in water or a true solution: it may be, a solution of oil in an aqueous solution of water-soluble constituents[1], or (*b*) a suspension of water in oil. If the oil is a suspension of water in oil, a large amount of water might be present without a significant conductivity being detected. In view of the concordance of the conductivity determinations with the relative turbidities, it appears probable that most essential oils contain oil either in aqueous suspension or in water. The behaviour of turpentine is singular, and it is therefore suggested that, in so far as a turpentine-water mixture tends to form a suspension, water is suspended in the turpentine. Parry [1925] makes a sharp distinction between conifers, in which the oil ducts are lysogenic, and the Rutaceae and many other plants in which the ducts are schizolysogenic or of mixed origin.

SUMMARY.

The classical work on absorption of radiant heat by the vapour of essential oils is in error owing to neglect of the water content of the latter. The effect is chiefly due to water vapour and only secondarily to the odoriferous constituents.

The experimental part of this work was carried out at the Northern Polytechnic, London. The author is glad to express his thanks to Mr J. Nicol, Head of the Physics Department, and to Dr T. J. Drakeley, now Principal, but then Head of the Chemistry Department, for much kindly interest and assistance. To Mr V. Hinkley especially, and also to Mr F. Avey, the author acknowledges considerable indebtedness for assistance in the design and construction of the forms of apparatus used. Messrs R. C. Treatt and Co., Ltd., of 11, Hart Street, London, E.C. 3, gave sympathetic co-operation in the selection and supply of genuine essential oils. Finally, the author wishes to thank Dr H. G. Thornton, of Rothamsted Experimental Station, for valuable criticism of the manuscript.

[1] This may not appear clear at first reading so an analogy is offered. If clove oil, containing substances soluble and insoluble in aqueous sodium hydroxide solution, is shaken with that solution the apparent solubility is frequently greater than the true solubility. This is due to substances dissolving in the resulting phenolate solution which were not soluble in sodium hydroxide before the phenols were taken up by the soda. In the oils referred to in the text, a water solution of part of the oil (*e.g.* of the lower alcohols) may dissolve more of the remaining oil than would pure water.

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LXXXIV. THE OXIDATION OF VITAMIN A *IN VITRO*. INFLUENCE OF THE SOLVENT.

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The problem of the oxidative destruction of vitamin A attracted the attention of the earliest workers upon the fat-soluble vitamins and has retained its interest on account of its bearing upon methods of handling and preserving materials containing vitamin A. Observations on the oxidation of vitamin A have generally been made at high temperatures, with the result that at first some confusion arose between the effects of heat and of oxygen upon the vitamin. A wide divergence of opinion among the early workers resulted [*cf.* McCollum and Davis, 1915; Osborne and Mendel, 1916; Steenbock, Boutwell and Kent, 1918], some holding that heat alone and others that oxidation alone was the cause of the observed destruction of the vitamin. This confusion was cleared up on the simultaneous appearance of papers by Hopkins [1920] and Drummond and Coward [1920]. Hopkins confirmed the findings of Osborne and Mendel [1916] that the vitamin A contained in butter is "relatively resistant to heat...but this factor is rapidly destroyed by exposure to atmospheric oxygen at temperatures from 15° to 120°." The same conclusion was reached by Drummond and Coward using different experimental methods. Thus it was definitely established that under certain conditions high temperatures have little destructive effect upon vitamin A in the absence of oxygen, and that oxidation actively destroys the vitamin both at high and low temperatures.

It was not possible at that time to obtain more than a qualitative demonstration of the oxidative destruction of vitamin A because the only available method for the estimation of the vitamin was the biological assay, and to follow the course of the oxidation quantitatively by this assay would require very large numbers of animals. The first quantitative data concerning the oxidation were in consequence not obtained until a chemical method for the estimation of vitamin A had been introduced. Wokes and Willimott [1927], employing the arsenic trichloride and antimony trichloride reagents for the estimation, demonstrated the rapid destruction of the vitamin A of cod-liver oil when the oil was aerated at temperatures from 88° to 125°, and obtained a figure for the temperature coefficient of the oxidation. They also showed, in accord with the results of Hopkins and of Drummond and Coward described above, that the destruction of the vitamin at these temperatures

was very much slower when the cod-liver oil was kept in tubes loosely plugged with cotton wool, without aeration or agitation.

Since the appearance of the paper by Wokes and Willimott little mention of the oxidation of vitamin A in solution is to be found in the literature, but the oxidation of the vitamin when adsorbed by fine powders has attracted attention. Dunn [1924] observed that the vitamin A of cod-liver oil was destroyed by mixing the oil with granulated starch and storing it in dark corked bottles. Marcus [1931] extended this observation by mixing a vitamin A concentrate with various fine powders and following the destruction of the vitamin by the antimony trichloride estimation. His results were in harmony with the idea that the destruction observed was due to oxidation of the vitamin by occluded oxygen at the surface of the particles, although the evidence is by no means conclusive.

At the present time it is generally agreed that although vitamin A is not destroyed by heat alone it is very labile and very readily oxidised. The writer has already shown in a preliminary communication [Dann, 1931] that this opinion requires strict qualification. Full details of the observations leading to this view are presented below.

Evidence that vitamin A is not always labile and easily oxidised. Smith and Hazley [1930] during an investigation of the antimony trichloride reaction of vitamin A were led to state that "It appears that the chromogen is more stable than has sometimes been supposed," although they failed to realise that it can resist the action of oxidising agents under certain conditions. There has however long been evidence of the considerable stability of vitamin A under some conditions in the recognition that saponification of liver oils followed by ether extraction of the soap solutions in the preparation of vitamin A concentrates causes little or no destruction of the vitamin. This demonstrates its stability (*a*) in alkaline alcoholic solutions at high temperatures, and (*b*) when dissolved in ether, which is an active oxidising agent unless specially purified immediately before use.

Cady and Luck [1930] found that the vitamin A of cod-liver oil shows marked stability and is not rapidly oxidised when treated with hydrogen peroxide. They emulsified cod-liver oil in an aqueous solution of hydrogen peroxide and observed that after 18 days at room temperature only partial oxidation of the vitamin A had occurred. Evidence of the stability of the vitamin A contained in butter is afforded by the work of Scheunert [1931] who demonstrated that a large fraction of the vitamin A remains intact during the ordinary processes of baking and roasting.

Factors influencing the oxidation of vitamin A in vitro. In addition to the well-recognised factors affecting the oxidation of vitamin A there is at least one other which appears to have escaped recognition: namely, the solvent effect. When for instance observations concerning the oxidation of the vitamin A contained in cod-liver oil have been made, it appears generally to have been assumed implicitly that the oxidation would follow the same course

had the vitamin A been contained in any other natural foodstuff or laboratory solvent, or even had it been separated in the pure state. Little attention has been paid to the influence exerted on the oxidation of the vitamin A by the complex media containing the vitamin, presumably owing to the implicit assumption mentioned above. As most of the media containing vitamin A are complex mixtures of fats which themselves undergo autoxidation, they may *a priori* be expected to exert an effect on the oxidation of the vitamin itself [*cf.* Moureu and Dufraisse, 1926].

For the related compound carotene Olcovich and Mattill [1931] found that when it occurs in vegetable foodstuffs its stability towards oxygen "does not depend upon the carotene itself or on the presence of oxygen, primarily, but is conditioned by the presence of other substances." It may be expected that the same will hold true for vitamin A.

As pure vitamin A is not yet available, data concerning the effect of chemical environment upon the oxidation may be obtained by dissolving a vitamin A concentrate in different solvents and following the oxidation of the vitamin in the solutions under similar conditions. When dilute solutions of the vitamin concentrate are used, it is to be expected that the effect of the impurities in the concentrate upon the course of the oxidation will be outweighed by the effect of the solvent, and that differences in the oxidation of the vitamin in different solvents can be referred to the effects of the solvents themselves.

EXPERIMENTAL.

A. Observations on the oxidation of vitamin A based on colorimetric determination[1].

1. *The oxidation of vitamin A by aeration in different solvents.*

The source of vitamin A for all the experiments described in this section was a crude cod-liver oil concentrate which gave a colour reaction of about 100 blue units per mg. with the antimony trichloride reagent. In following the course of oxidation of the vitamin in a given solvent the following procedure was adopted throughout. About 150 mg. of the concentrate were transferred to a 100 cc. round-bottomed flask and weighed. The calculated weight of solvent was added to produce a solution containing 1 % by weight of the concentrate, and the flask warmed and shaken until the concentrate had dissolved. A 1 cc. sample of the solution was withdrawn by pipette and its vitamin A content measured colorimetrically. The flask was fitted with a stopper bearing a reflux condenser and a narrow inlet tube reaching to the bottom of the flask; the condenser was clamped in position over a water-bath so that the boiling water reached the neck of the flask. Through the inlet tube was passed a quick current of air which had been dried in a calcium chloride tube and filtered through glass-wool. At intervals a 1 cc. sample of the solution

[1] The results of the colorimetric determination of vitamin A are expressed in terms of "blue units" as defined by Moore [1930].

was removed by pipette and its vitamin content estimated. All experiments were performed with the aeration flask in a boiling water-bath and observation showed that the temperature of the solutions remained at 98°, except when using ethyl alcohol and ethyl acetate as solvents, when the solutions remained at 78° and 66° respectively.

In order to estimate the vitamin content of each sample of solution, the vitamin was separated from the solvent as described below and dissolved in 2 cc. of chloroform. An aliquot part of the chloroform solution was diluted to 0·5 cc. with chloroform, 2 cc. of the antimony trichloride reagent were added and the colour of the fluid was matched against the standard glasses of the Lovibond tintometer. A standard tube, 1 cm. diameter, was used to

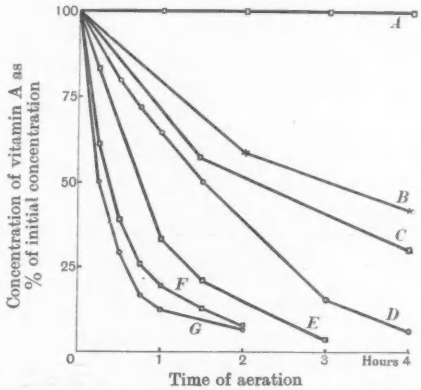

Fig. 1. The oxidation of vitamin A when dissolved in esters and aerated at 98°. *A*. Ethyl acetate and triacetin. (Ethyl acetate at 66°.) *B*. *n*-Amyl acetate. *C*. Tributyrin. *D*. Triolein. *E*. Ethyl laurate. *F*. Ethyl oleate. *G*. Ethyl stearate.

hold the reaction mixture, and the dilution of the solution of vitamin arranged so that the resultant colour with antimony trichloride was matched by 3–5 Lovibond blue units. The procedure adopted to separate the vitamin from the solvent varied for different samples according to the solvent. (*a*) For solutions in esters, the sample was run into 10 cc. of 20 % alcoholic potash and boiled for 3 minutes. The resultant solution was poured into water and the vitamin extracted from the soap solution in the usual way with ether. (*b*) For solutions in acids, the sample was run into 40 cc. 5 % aqueous potash, shaken to dissolve the acid, and the vitamin separated by ether extraction. (*c*) For solutions in alcohols, the sample was diluted with chloroform and the antimony trichloride test applied direct, after it had been found by trial that the alcohols used had no effect upon the colour test. For solutions in ethyl alcohol and alcoholic potash the samples were run into 40 cc. water and the vitamin extracted by ether.

The experimental results are presented graphically. Unsmoothed curves

obtained in single experiments are given as it was found possible to repeat them within the limits of experimental error. In each figure the percentages of the initial vitamin contents of the solutions remaining undestroyed during aeration are plotted as ordinates against the times of aeration as abscissae.

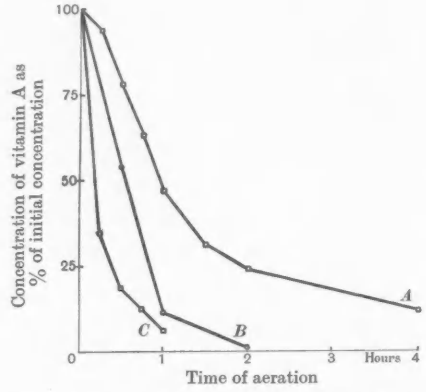

Fig. 2. The oxidation of vitamin A when dissolved in acids and aerated at 98°. *A*. Caproic acid. *B*. Acetic acid. *C*. Stearic, oleic and lauric acids.

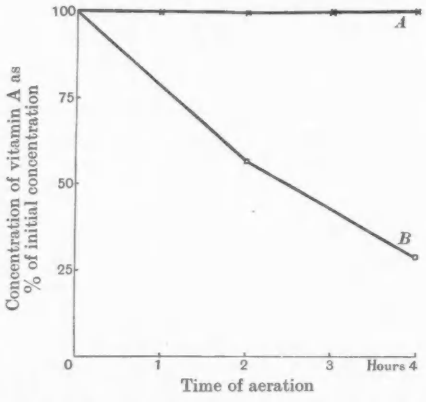

Fig. 3. The oxidation of vitamin A when dissolved in alcohols and aerated at 98°. *A*. Ethyl, *n*-butyl and cetyl alcohols. (For ethyl alcohol the temperature was 78°.) *B*. *n*-Amyl alcohol.

In Fig. 1 are collected the results obtained with a number of esters as solvents, and it is evident that there is a great variation in the rate of oxidation of vitamin A when aerated under similar conditions in solutions of different esters, from an oxidation too slow to be measured during 4 hours in ethyl acetate and triacetin to an oxidation so rapid as to be almost complete within 2 hours in ethyl oleate and ethyl stearate. The variation in the rate of oxida-

tion of the vitamin when aerated in different acids is much less; oxidation is relatively rapid in all the acids (Fig. 2). In the alcohols examined, on the contrary, the rate of oxidation is always relatively slow; for three out of the four no measurable oxidation occurred in 4 hours (Fig. 3). For the miscellaneous solvents, no measurable oxidation took place in 20 % alcoholic potash

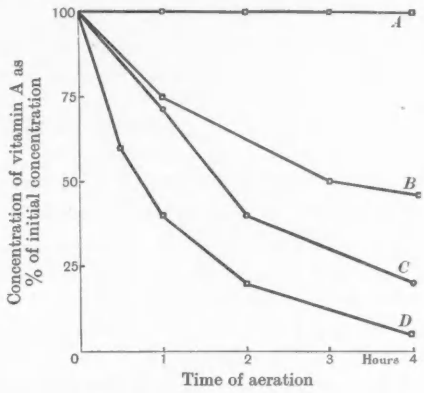

Fig. 4. The oxidation of vitamin A when dissolved in various solvents and aerated at 98°. *A*. 20 % alcoholic potash. *B*. Cod-liver oil. *C*. Coconut oil. *D*. Arachis oil.

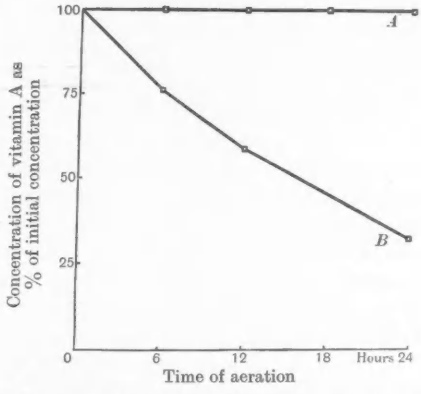

Fig. 5. The oxidation of vitamin A dissolved in different solvents and aerated at 98°. (For ethyl alcohol at 78°.) *A*. Ethyl alcohol and ethyl alcoholic potash. *B*. Cetyl alcohol.

within 4 hours, and fairly rapid oxidation in the natural oils (Fig. 4). The observations upon ethyl alcohol, cetyl alcohol and 20 % alcoholic potash were repeated over an extended period of 24 hours; no oxidation of vitamin A could be measured after this time when aerated in ethyl alcohol or alcoholic potash, but a considerable oxidation occurred in cetyl alcohol (Fig. 5).

The observations recorded above and included in Figs. 1–5 were all made with a single specimen of vitamin A concentrate of rather low potency (1 mg. gave 100 blue units with antimony trichloride) which was chosen as it had remained unchanged in strength over a period of months. These experiments were almost all repeated using a second specimen of concentrate of approximately the same potency but obtained from a different source, with results which were identical within the limits of experimental error.

Some observations of oxidation by aeration were also made using high potency concentrates as source of vitamin A, but the number of experiments performed was limited by the small amount of the concentrates available, and they were therefore confined to the use of one solvent. Ethyl alcohol was chosen on account of the interest of the earlier results with this solvent. Two concentrates were treated, one prepared from pig-liver oil (1 mg. gave 1000 blue units) and the other from turbot-liver oil (1 mg. gave 1250 blue units), and with each specimen no oxidation occurred when the vitamin was dissolved in ethyl alcohol and aerated at the boiling-point for 24 hours.

A further observation with alcohol as solvent was made at a lower temperature using a rat-liver oil concentrate (1 mg. gave 2150 blue units) as source of the vitamin. 100 mg. of the concentrate were dissolved in 10 cc. ethyl alcohol and the solution was stored in a conical flask loosely plugged with cotton-wool, kept at 0°. At intervals 1 cc. samples of the solution were withdrawn for estimation of the vitamin concentration and it was found that after 4 months 22 % of the vitamin A had disappeared. Reasons are given below, however, for believing that the observed destruction of vitamin A in this experiment was not oxidative.

2. *The aeration of solutions of cholesterol.*

It has been shown [Takahashi *et al.*, 1925] that the rather ill-defined substance called oxycholesterol responds somewhat like vitamin A to a number of chemical tests, and in particular [Rosenheim, 1927] it reacts with antimony trichloride to give a blue coloration. As the vitamin A concentrate used in most of the experiments described above contained some cholesterol, the question of possible interference by oxycholesterol was investigated. If oxycholesterol were formed during the aeration of a solution of vitamin A, the observed rate of oxidation of vitamin A would be less than the true rate of oxidation. Blix and Löwenhielm [1928] found that pure cholesterol is not transformed into oxycholesterol when dissolved in ethyl alcohol and aerated for a few hours at 60°–70°, but it was thought desirable to extend this observation.

0·2 g. cholesterol was dissolved in 10 cc. ethyl alcohol and aerated at the boiling point of the solution for 24 hours. 5 cc. of the solution were evaporated to dryness and the residue was dissolved in 2 cc. chloroform and tested by adding 0·5 cc. of the solution to 2 cc. of antimony trichloride reagent. No colour was produced. Thus although the test sample contained at least a

hundred times as much cholesterol as any of the samples taken in the oxidation experiments described in the last section, no perceptible amount had been transformed into oxycholesterol. This experiment was repeated with 1 % of ethyl oleate dissolved in the alcohol together with the cholesterol, with identical results. As further evidence, the observations upon aeration of vitamin A dissolved in ethyl alcohol, *n*-butyl alcohol and ethyl acetate were repeated with 1 % cholesterol added to the solutions. The results were identical with those obtained without the added cholesterol.

Thus the observed stability of vitamin A when aerated in certain solvents cannot be ascribed to oxidation of the cholesterol to oxycholesterol capable of exerting a compensatory effect in the colorimetric estimation.

3. *The effect of hydrogen peroxide upon vitamin A in alcohol.*

The remarkable stability of vitamin A in alcohol to atmospheric oxygen suggested the extension of the observations to some other oxidising agent. Cady and Luck [1930] showed that the vitamin A of cod-liver oil is attacked slowly by hydrogen peroxide in the cold, but a possible criticism of their results may be directed at the ill-defined conditions under which the reaction was studied. The vitamin and the hydrogen peroxide were present in separate liquid phases whose degree of dispersion was unknown and changing. Alcohol as solvent has the advantage that it dissolves both vitamin A and hydrogen peroxide and observations can therefore be made on a homogeneous solution. The following experiment was performed. 137 mg. of a vitamin A concentrate were dissolved in 9 cc. of alcohol and 1 cc. of aqueous hydrogen peroxide (30 % H_2O_2 by weight) was added. After stirring, 1 cc. of the reaction mixture was withdrawn by pipette and run into 25 cc. ether. The ethereal solution was washed with water, dried and evaporated; the residue was dissolved in chloroform and its vitamin content estimated. The reaction mixture was allowed to stand at 15° and at intervals further samples were taken. At the end of 24 hours the vitamin content of the solution remained unchanged. Therefore under the conditions described no oxidation of vitamin A by hydrogen peroxide takes place.

B. Observations on the oxidation of vitamin A based on the biological assay.

1. *The stability of vitamin A in alcohol verified by biological tests.*

Although it is now realised that for dealing with concentrates the colorimetric estimation of vitamin A with the antimony trichloride reagent is quite trustworthy, it is still desirable to check any new result by applying the biological assay for confirmation. In the present study it was out of the question to use the biological assay to follow the oxidation of vitamin A in more than one or two solvents even had this been thought necessary, owing to the large number of animals which would be required. The more important

results obtained with the colorimetric estimation were therefore selected for confirmation with the biological assay. The stability of vitamin A in alcohol when aerated at its boiling point or treated with hydrogen peroxide at room temperature, and its stability when aerated in 20 % alcoholic potash at 98° were chosen for repetition. Stability of the vitamin under these conditions was demonstrated by preparing solutions in coconut oil of untreated and of treated concentrates, and comparing the vitamin A contents of these solutions by feeding tests. The solutions were prepared as follows.

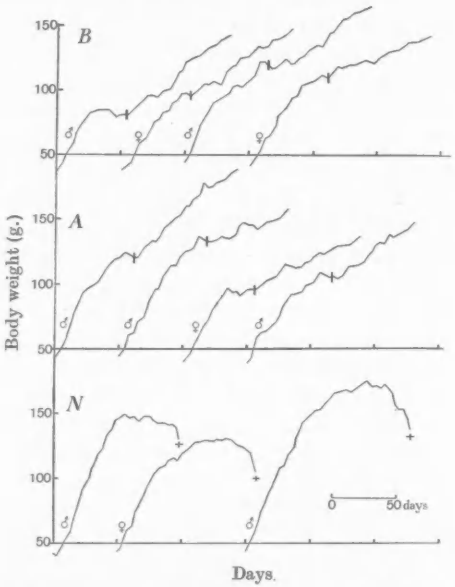

Fig. 6. Biological confirmation of results obtained colorimetrically. *N*. Negative controls. *A*. Animals receiving untreated vitamin A concentrate. Daily dose per rat, 3 drops solution A, containing 0·05 mg. concentrate. *B*. Animals receiving vitamin A concentrate which had been aerated in boiling ethyl alcohol for 24 hours. Daily dose per rat, 3 drops solution B, containing 0·05 mg. concentrate.

Solution A. Solution of untreated concentrate, prepared by dissolving the vitamin A concentrate, 1 mg. to each gram of coconut oil.

Solution B. Solution of vitamin A concentrate aerated in alcohol. 190 mg. of the concentrate were dissolved in 19 cc. alcohol and aerated at the boiling point for 24 hours. The alcohol was evaporated under reduced pressure and the residue dissolved in 190 g. of coconut oil.

Solution C. Solution of vitamin A concentrate aerated in alcoholic potash. 80 mg. of concentrate were dissolved in 10 cc. of 20 % alcoholic potash and aerated at 98° for 24 hours. The solution was diluted with water and the vitamin extracted and dissolved in 80 g. coconut oil.

Solution D. Solution of vitamin A concentrate treated with hydrogen peroxide. 40 mg. of concentrate were dissolved in 10 cc. alcohol and 1 cc. of aqueous hydrogen peroxide (30 % H_2O_2 by weight) was added. The mixture was shaken and allowed to stand 24 hours at room temperature, after which the vitamin was extracted (see above) and dissolved in 40 g. of coconut oil.

Previous observation with the untreated concentrate had shown that if 3 drops of solution A were given daily to a rat deprived of vitamin A, submaximal growth was maintained. A group of matched albino rats was placed

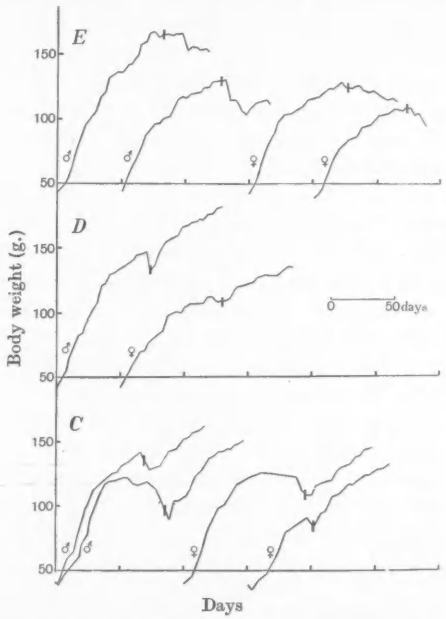

Fig. 7. Biological confirmation of results obtained colorimetrically. *C.* Animals receiving vitamin A concentrate which had been aerated in alcoholic potash at 98° for 24 hours. Each rat received 3 drops solution C daily (containing 0·05 mg. concentrate). *D.* Animals receiving vitamin A concentrate which had been treated with hydrogen peroxide. Daily dose per rat, 3 drops solution D, containing 0·05 mg. concentrate. *E.* Animals receiving vitamin A concentrate which had been aerated in ethyl stearate at 98° for 4 hours. Daily dose per rat, 5 drops of solution E, containing 0·27 mg. concentrate.

on a vitamin A-free diet made up of 694 g. rice starch, 200 g. Glaxo "vitamin A-free casein," 50 g. salts, 1·1 cc. radiostol and 50 g. marmite. After depletion of its vitamin A reserves each animal was dosed daily with 3 drops of one or other of the test solutions. Four animals received untreated concentrate (solution A), four received concentrate aerated in alcohol (solution B), four received concentrate aerated in alcoholic potash (solution C), two received concentrate treated with hydrogen peroxide (solution D) and three others received no dose. The growth curves are given in Figs. 6 and 7 and it will

be seen that all four of the solutions were equally efficient in promoting growth. Within the limits of error of this test (which will be large owing to the small number of animals used) the solutions contain equal amounts of vitamin A, indicating the stability of the vitamin when exposed to oxygen or hydrogen peroxide under the conditions described, and confirming quantitatively the results obtained by colorimetric estimation.

2. *Oxidation of vitamin A by aeration in ethyl stearate.*

An observation was made to confirm the rapid oxidation of vitamin A when aerated in certain solvents; ethyl stearate was chosen as the solvent. A test solution of vitamin concentrate which had been aerated in ethyl stearate was prepared.

Solution E. 70 mg. of the concentrate were dissolved in 7 g. ethyl stearate and aerated at 98° for 4 hours. The solution was saponified and the residue dissolved in 21 g. coconut oil. This solution was given in a daily dose of 5 drops to each of four rats taken from the same group as those used in testing the other four solutions. The growth curves are reproduced in Fig. 7, and it will be seen that the dose of 5 drops was insufficient to support growth or even maintain the weight of the rats. As 5 drops of solution E contains 50/9 as much concentrate as 3 drops of solution A (untreated concentrate) it follows that the concentrate after aeration in ethyl stearate contained much less than 18 % of its initial content of vitamin A. The colorimetric test showed that aeration for the same period under the same conditions reduced the vitamin content of an ethyl stearate solution to 3 % of the initial value. In this experiment therefore the biological test affords a qualitative confirmation of the results obtained by the colorimetric method of estimation.

Discussion.

The experiments described above establish definitely that the solvent plays a cardinal part in determining the rate of oxidation of dissolved vitamin A by atmospheric oxygen. This is well illustrated by the data showing the percentage of the vitamin which is oxidised in one hour under similar conditions

Table I. *The percentage of dissolved vitamin A which is destroyed by aeration for one hour at 98°.*

Solvent	% oxidised	Solvent	% oxidised
Ethyl alcohol*	0	Ethyl laurate	67
n-Butyl alcohol	0	Ethyl stearate	87
n-Amyl alcohol	22	Ethyl oleate	81
Cetyl alcohol	0	*n*-Amyl acetate	23
Acetic acid	89	Triacetin	0
Caproic acid	54	Tributyrin	31
Lauric acid	93	Triolein	36
Stearic acid	94	Coconut oil	29
Oleic acid	92	Arachis oil	60
Ethyl acetate†	0	20 % alc. KOH	0

* At 78°. † At 66°.

in different solvents. No correlation between the rate of oxidation of vitamin A and the molecular constitution of the solvent is apparent although it is perhaps significant that the rate is great in all the acids tried, while it is negligible in some alcohols and esters and also in strong alcoholic potash. Similarly no correlation between the rate of oxidation and the solubility of oxygen in the solvent can be discerned.

The work of Huston, Lightbody and Ball [1928] and of Mattill [1931] suggests that the phenomena of autoxidation may play a part in determining the stability of the fat-soluble vitamins, but at the present time the theory of autoxidation cannot be applied to the elucidation of the detailed results reported above. Until observations can be made upon the oxidation of pure vitamin A dissolved in rigorously purified solvents, this must inevitably remain true, as the effects of traces of impurities cannot be predicted. Any substance present in traces might exert a pro-oxidant or an anti-oxidant effect upon the oxidation of the vitamin A; moreover a single substance is able to act as pro-oxidant under some conditions and anti-oxidant under others [*cf.* Moureu and Dufraisse, 1926].

The results (obtained with the ordinary grade compounds easily available in the laboratory) bear directly upon questions concerning the concentration and preservation of vitamin A preparations. For instance, it is evidently superfluous to take special precautions to prevent the oxidation of vitamin A during saponification processes, although this has been considered necessary [Drummond *et al.*, 1925; Smith and Hazley, 1930]. The effect of ethyl alcohol in protecting vitamin A from oxidation has already been applied successfully in the treatment of concentrates. Although they deteriorate rapidly in air, if dissolved in ethyl alcohol they appear to escape oxidation completely for days or weeks, without special precautions, and only lose their potency slowly over a period of months. Moreover, some observations on the colour change which occurs in alcoholic solutions of vitamin A when kept for several months suggest the possibility that the destruction of the vitamin in this solvent may not be due to oxidation, but to isomerisation. Further work upon this question is now in progress.

Summary.

Vitamin A is rapidly oxidised by air when dissolved in some solvents, and slowly or not at all in others. It is particularly stable in ethyl alcohol, alcoholic potash and ethyl acetate. In ethyl alcohol it is also stable toward hydrogen peroxide.

The solvent (or impurities associated with the vitamin or solvent) appears to play a leading part in determining the stability of vitamin A.

I wish to express my indebtedness to Dr L. J. Harris and Dr T. Moore for much help and advice; also to the Medical Research Council for the tenure of a part time grant during the progress of this work.

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LXXXV. A STOCK DIET FOR RATS BRED FOR VITAMIN TESTS.

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The diet of rats bred for vitamin tests should be rich enough in all dietary factors to allow normal growth of the young and free reproduction; but it must not be so rich that the young selected for experimental work do not exhaust their reserves of vitamins in a reasonable time when given vitamin-deficient diets. It is desirable also that there should be as little difference as possible between the reserves of members of the same litter and of different litters. It is necessary then, that the stock diet should vary as little as possible from day to day and also from season to season. The ideal diet for this purpose will only be available when all dietary essentials have been recognised and when they can be procured in a pure state. In the meantime a stock diet must be determined largely by trial of materials available in a reasonably stable and uniform state.

We have obtained evidence that a diet made from a given recipe may be satisfactory in one laboratory but unsatisfactory in another laboratory, although it may be very difficult to determine wherein the respective constituents of the diets differ. Kon [1931] has had a similar experience. After failure to improve two diets found to be satisfactory in various laboratories, but unsatisfactory in our own, we have devised a third one which has proved adequate for growth, reproduction and lactation of the colony. Evidence that the young rats of this colony are suitable for tests on vitamins A and D is given.

Diet A.

When the vitamin work of these laboratories was begun in 1927, it was decided to adopt the stock diet of the Wisconsin University laboratories of which one of the writers (K. H. C.) had had 10 months' working experience in 1925–6. The diet [Steenbock, 1923] had been in use there for about 9 years practically without modification. It consisted of:

Yellow corn (ground)	76·0 %
Linseed oil meal	16·0 ,,
Crude caseinogen	5·0 ,,
Ground alfalfa	2·0 ,,
Sodium chloride	0·5 ,,
Calcium carbonate	0·5 ,,

Table I. *The influence of various stock diets on the powers of reproduction of rats.*

Diet	No. of does on diet	Diet of parents	No. of litters Born	No. of litters Weaned	No. of rats weaned	Av. wt. of rats weaned per doe g.
1. Mixed diet from weaning, diet A from birth of first litter	12	Mixed	67	58	335	843·8
2. A from weaning	9	Mixed, A from birth of first litter	26	13	77	148·5
3. A from weaning, B after first litter	17	A from weaning	33	2	12	15·9
4. B from weaning, 5 % dried yeast added after birth of first litter	1	A from weaning	2	0	0	0
5. B from weaning, watercress added after birth of first litter	4	Mixed from weaning, A from birth of first litter	5	0	0	0
6. A from weaning, mixed before birth of first litter	6	Mixed	10	5	21	78·5
7. A + watercress	8	Mixed	15	3	7	19·4
8. B + 5 % dried yeast + watercress	3	Mixed	7	5	25	176·7
9. B + 5 % dried yeast + watercress and "light white casein" in place of skimmed milk	3	Mixed	8	5	28	254·7
10. A + 10 % "light white casein" + watercress	2	Mixed	4	0	0	0
11. A + 10 % dried yeast + watercress	2	Mixed	6	2	6	53
12. A + 8 % dried yeast + watercress + 10 % "light white casein"	2	Mixed	7	3	16	147
13. A	4	Mixed	5	0	0	0
14. A irradiated	5	Mixed	4	1	4	24
15. A + 0·04 g. cod-liver oil daily	3	Mixed	1	0	0	0
16. A + 10 % dried yeast	2	Mixed	3	3	13	170
17. A + 10 % "light white casein"	2	Mixed	3	0	0	0
18. A (1930) test	11	C, L, W, P	16	6	28	63·5
19. A + 4 g. lean beef per rat per week	13	C, L, W, P	15	5	28	48·7
20. C + liver and watercress	19	Mixed	58	48	252	441·8
21. C + liver and watercress	7	Mixed different source	18	11	44	63
22. C + liver and watercress	6	Do.	19	14	60	297·8
23. C + liver and watercress	B and W 5	Mixed, then B from birth of first litter	13	12	64	371
24. C + liver and watercress	11	C, L and W	21	12	56	141·3
25. C and porridge after weaning of first litter, mixed previously	8	Mixed	28	23	137	521·5
26. C and porridge from about 10 weeks of age (the first litters from 25)	37	Meal ration, green food, varying amounts of milk	89	44	208	151·9
27. C and porridge (second or subsequent litters from 25)	12	Do. till weaning of first litter, then C and P	47	28	123	278·2
28. C + liver and watercress with extra 5 % dried yeast and extra 6 % "light white casein"	8	C and porridge	31	27	140	474·4
29. Do.	3	Do.	17	12	65	533
30. Do.	B and W 3	C, L, W, P	18	11	53	505·7
31. Do.	3	Mixed	14	7	40	304·7
32. C + L, W and P, 1st generation	B and W 92	C, some P, some L, some W, some any two, none all three	280	205	1144	416·2
33. C + L, W and P, 2nd generation	71	C, L, W, P	219	137	768	309·7
34. C + L, W and P	21	C, L, W and mostly P	56	40	212	296·7
35. C + L, W and 20 % dried milk	21	Do.	72	56	321	426·6
36. C + L, W and P	12	Mixed	69	49	230	651·0
37. C + L, W and P, 1st generation	B and W 27	C, L, W	51	29	153	159·4
38. C + L, W and P, 2nd generation	B and W 22	C, L, W, P	56	28	131	170·4
39. C + L, W and 20 % dried milk	B and W 134	C, L, W, P or C with 20 % dried milk, L and W	458	310	1769	361·6*
40. High vitamin	30	C, L, W, P	40	24	122	116·8

* Nine of these does are still alive.

In addition to unlimited quantities of this mixture the rats were also given unlimited quantities of fresh milk from cows which were kept specially for the work on a winter feed at the University farm. For the young does, 5 % of butter was added to the diet, but for the older does (those which had already borne three litters) this was omitted.

In January 1927, this recipe, spoken of as diet A in this paper, was used for the first rats imported into these laboratories. Four bucks and twelve does (two litters) were obtained from the Glaxo Research laboratories. They were pure albinos of the Wistar strain, and all the does were either pregnant or suckling their first litters, the result of brother-sister mating. The result is shortly summarised in Table I, 1. The rats which had grown up on a mixed diet in the Glaxo laboratories throve on the new diet and in time produced 5–6 litters each of good average weight. But, as certain of these litters were selected for breeding and allowed to grow up, it became evident that the later litters were growing more slowly than the earlier ones (Table II). Moreover,

Table II. *The decreasing rate of growth of successive litters taken for breeding from rats fed on diet A.*

Date of selection of litter for breeding	Litter	Weight at 12 weeks (g.) ♂	Weight at 12 weeks (g.) ♀
17. iii. 27	Second from ♀ 15	204	149
			151
			141
19. iii. 27	,, ♀ 6	197	158
			160
			147
5. iv. 27	,, ♀ 8	187	144
			144
			148
7. iv. 27	,, ♀ 10	177	135
			133
			118
13. iv. 27	,, ♀ 2	171	134
		171	131
			133
			131
14. v. 27	Third from ♀ 6	155	133
			140
			136
			137
			124
25. v. 27	,, ♀ 16	169	131
			139
			131
			132
			131

as these litters grew up and reproduced, the number of litters weaned and the weight of each litter weaned became less and less (Table I, 2). It was painfully evident that the diet, as made with materials available in this laboratory, was inadequate for normal growth and reproduction.

Various experiments were then carried out to try to discover the deficiency. Litters of young rats freshly imported into the laboratories were divided into groups so that each litter was represented in each group. The groups were then fed from about the age of 4 weeks till death on some modification of diet A. The results are summarised in Table I, 10–17. The only improvement in the diet was that made by the addition of 5 % dried yeast, but even with this addition, it was obvious that some factor or factors other than those contained in dried yeast was lacking. The addition of extra protein in the form of 10 % "light white casein," or of extra vitamin D by the irradiation of the diet, or of extra vitamins A and D by the giving of 40 mg. cod-liver oil (directly by mouth) each day did not effect any improvement. A recent experiment (1930, when other diets were giving good results) showed diet A still to be inadequate and not improved by the addition of about 4 g. lean beef per rat per week (Table I, 18 and 19).

Fresh tap-water is always supplied daily in inverted narrow-necked bulbs. Bedding consists of sawdust, and wood-wool is supplied to each doe when she is isolated a few days before the birth of a litter.

Each rat or group of rats is given a clean cage at least once a week. The dirty cages are scraped and washed with hot water, occasionally also with soap.

The colony is housed in a room with a north aspect. It is heated by one hot water radiator and two electric ones controlled by a thermostat so that the temperature remains nearly constant at about 70° F. Ventilation is arranged through door and window. There is no scabies in this colony.

Diet B.

Meantime a smaller experiment had been made with the Sherman diet referred to as diet B in this paper [Sherman and Campbell, 1924]. It consisted of

Whole wheat (ground) with 2 % NaCl	67 %
Dried whole milk (or skimmed milk with proportionate butter)	33 „

From Table I, 3–5, it may be seen that this also was unsatisfactory. Experiments (Table I, 8 and 9) were made to try to improve the diet by the addition of watercress and 5 % dried yeast and also by the substitution of "light white casein" for the dried skimmed milk. The young born from rats in these experiments were few in number and grew at a subnormal rate. No further attempts were made to improve either of these diets A and B, although some of the does which had been fed on one or other were later fed on a diet of kitchen scraps to see whether they were still capable of reproducing freely if properly fed (Table I, 6). No litters were produced.

Throughout the whole of this work, so far, the oestrous cycles of the rats had been examined by daily examination of the vagina from the time when they had reached a suitable weight for mating (about 140–150 g.). The does

were mated only at the cycles, *i.e.* when the contents of the vagina had been free from leucocytes and contained only nucleated and cornified cells.

With the rats which had been imported into this laboratory when mature, it was very seldom that copulation did not take place at the mating, and no instance of foetal resorption was noted, which would have been indicated by failure to produce a litter after red blood cells had been found in the vagina 14 days after copulation. With the young rats that grew up from our own breeding, cycles occurred regularly and copulation took place at mating, but very frequently this was not followed 14 days later by the finding of red blood cells in the vagina. For example one doe received a buck (sperm found in vagina) on 13 different occasions but only once produced a litter; this litter died within 4 days of birth. Brother-sister mating, and cross-mating were equally ineffective. Three healthy bucks of proven fertility were imported from the Glaxo Laboratories but were no more effective than the bucks from our own breeding. Hence it appeared certainly to be an impoverishment of the does and possibly also of the bucks.

A point of interest emerged at this stage of the breeding. In Table III it is shown that the longer the period of gestation the less chance is there of the litter being weaned.

Table III. *The correlation between the mortality of young rats and the length of their gestation period.*

Gestation period (days)	Litters born	Litters which lived at least 1 week	Litters weaned	% litters which lived at least 1 week	% litters weaned
21	48	40	35·5	83	74
22	206	139·5	116	68	56
23	76	40	30	53	39
24	20	8	7	40	35
25	7	2	1	29	14
26	1	0	0	0	0

Diet C.

Meanwhile, another diet (named the C stock diet, as it was the third one to be used in this laboratory) had been devised which for some time promised to give good results. It consisted of:

Yellow maize, ground	65 %
Whole wheat, ground	20 ,,
"Casein, light white" (B.D.H.)	9 ,,
Dried yeast	5 ,,
Sodium chloride	0·5 ,,
Calcium carbonate	0·5 ,,

About 2 g. fresh liver and 2 g. fresh watercress were given to each rat twice a week. The result of this is seen in Table I (20–25). Reproduction was very much better than on the previous diets but still not satisfactory and a

modification of the diet was made. Since the addition of cod-liver oil, dried yeast and watercress to diet A had not given results equal to those obtained in the Wisconsin Laboratories, it seemed possible that the milk from the ordinary London supply was deficient in some hitherto unrecognised factor. As it was not easy to get a daily supply of fresh untreated milk, it was decided to try the C stock diet supplemented by porridge made with Plasmon oats (which contain some dried milk) mixed with the proportion of "fresh" milk usually taken by human beings. The liver and watercress were not given to the rats having the C stock diet and porridge. This diet was given to certain rats which had been sent to these laboratories together with their first litters, and which had previously been brought up on a diet of mixed meat, green food and varying amounts of milk. The result of this trial was more successful (Table I, 25). The first litters of these rats were fed on the C stock diet from about 4 weeks of age and on porridge also from about 10 weeks. The result was fairly bad (Table I, 26). Certain of the second and subsequent litters of the same rats were fed on the C stock diet and porridge from the time of weaning. The result was better (Table I, 27) but not so satisfactory as that obtained with the rats which had been brought up on a mixed ration and given diet C only after the weaning of the first litter.

Other modifications of the original C stock diet were also tried. A further 6 % "light white casein" (to increase the protein) and 5 % dried yeast (possibly required to balance the extra protein) were added and the usual quantities of liver and watercress were given. Good results were obtained (Table I, 28–31). Also, the combination of C stock diet, liver, watercress and porridge was tried on other litters. The result with the first generation of rats on this diet was satisfactory (Table I, 32) but when it became apparent that the second generation was not thriving on it as well as the first (Table I, 33), another attempt was made to improve it.

This was carried out as a definite experiment. Each of 14 litters was divided equally into two parts at 4 weeks of age. Half of each litter was brought up on C stock diet, liver, watercress and porridge and the other half was brought up on C stock diet containing 20 % dried milk, liver and watercress (no porridge). The result shows that the diet containing the dried milk is definitely better than the one that did not contain it but which was supplemented with porridge instead (Table I, 34, 35). Further litters on this modification (20 % dried milk added to the C stock diet, *plus* liver, *plus* watercress) also gave good results (Table I, 39).

Failure of black and white rats fed on diet C.

These tests were made almost wholly on albino rats. Our experience with a black and white strain of rats fed from weaning on C stock diet, liver, watercress and porridge is interesting. Litters from rats which had been brought up on a mixed diet reproduced well (Table I, 36). Litters from rats which had been brought up on C stock diet, liver and watercress (no porridge) did

much less well (Table I, 37), and the second generation did equally badly (Table I, 38). The result obtained with the black and white strain of rats was considerably worse than that obtained with the albino strain. The difference has not been investigated further. It is probable that the diet which is just good enough for the albino strain is not (somehow) good enough for the more vigorous black and white strain.

Vitamin-rich diet.

Still another trial was made to see how far the C stock diet could be improved by supplementing it with foods rich in vitamins. Nine litters were given the C stock diet in unlimited amount and, twice weekly, a double quantity of liver or beef, about 4 g. lettuce, 4 g. watercress and 3 g. hard-boiled egg, and, every day, porridge, about 0·5 g. marmite in water and 1 drop (0·02 g.) of a good sample of cod-liver oil. The result was distinctly poor. (Table I, 40 contrast with results 32–35 and 39. The parents of the three groups of rats had been brought up on the same diet C.)

Comparison of the main diets tried.

The results obtained from each of the main diets recently used are further summarised in Table IV in which are given (*a*) the average weight of a young

Table IV. *Summary of the more important results detailed in Table I.*

	Av. wt. of rat at 3 weeks g.	Av. No. of rats weaned per litter	Av. wt. of each litter at 3 weeks g.	Av. total wt. of young from each doe g.	% of litters weaned
Albino rats.					
C stock, liver and watercress	30·9	4·9	151·7	306·6	75·2
C stock, L, W and porridge: 1st generation	33·3	5·6	186·8	416·2	73·2
2nd generation	28·6	5·6	160·5	309·7	62·5
C stock, L, W and porridge } Litter com-	29·4	5·3	155·8	296·7	71·4
C stock, L, W, 20 % dried milk } parison	27·9	5·7	160·0	426·6	77·4
C stock, L, W, 20 % dried milk	27·3	5·7	156·1	361·6*	67·7
High vitamin diet	28·7	5·1	146·0	116·8	60·0
Black and white rats.					
C stock, L, W and porridge: 1st generation	28·1	5·3	148·4	159·4	24·2
2nd generation	28·6	4·7	133·8	170·4	50·0

* Nine rats still alive.

rat at 3 weeks, (*b*) the average number of rats in each litter weaned, (*c*) the average weight of a litter at 3 weeks, (*d*) the average total weight of young (3 weeks old) from each doe, and (*e*) the percentage of litters weaned.

We therefore decided to adopt the C stock diet with 20 % dried milk, liver or beef (see below) and watercress, twice a week for the whole of our colony; but being alarmed at the number of litters that died during lactation, we decided to add a further 5 % yeast to the diet of the lactating does. The result is seen in Table V. There is still a high mortality of first litters but,

Table V. *Diet. C stock* plus *20 % dried milk, liver and watercress.*

	No extra source of vitamin B			Marmite given from birth of young			5 % dried yeast (extra) from birth of young		
	No. of litters born	No. of litters weaned	% litters weaned	No. of litters born	No. of litters weaned	% litters weaned	No. of litters born	No. of litters weaned	% litters weaned
First litters	49	24	49	95	48	50·5	100	55	55
Second litters	64	22	34·4	2	2	100	100	63	63
Third litters	11	4	36·4	1	1	100	93	59	63·4
Fourth litters	12	8	66·7	—	—	—	49	39	79·6
Fifth litters	7	6	85·7	—	—	—	18	17	94·4
Sixth litters	—	—	—	—	—	—	9	6	66·7

judging only from the groups which contain a reasonably high number of litters, it would appear to be advantageous to give the extra dried yeast to the lactating does. During the third week of lactation, no liver is given in order to prevent any possibility of the young eating it and thus varying their reserves of vitamin A.

The mating of the rats.

During the work on the C stock diet and its modifications, does were mated (two or three to one buck) when they reached 140–150 g. in weight, and 2 to 3 weeks' rest was given after the weaning of a litter before the doe was again mated. Thus we can trace the parentage (male and female) of all the rats at present in the colony. Every doe was weighed once a week and, when she was obviously pregnant, she was given a separate cage. The young were weighed weekly and weaned when 23 days old. Now, however, having gained the knowledge we desired concerning a suitable diet, we are no longer keeping records of matings. We have divided our breeding rats into four groups according to the quarter of the year in which they were born. Mating is arranged within each group, pregnant does are given separate cages and the only weighings taken are those of the litters when 3 weeks old.

We examined the vagina of 47 of the rats on the C stock diet with 20 % dried milk, daily from the date of opening of the vagina until they were about 9 months old, though only on the fourteenth day after the finding of sperm and not at all during lactation. We found the occurrence of the oestrous cycle normal but we have many times found sperm in the vagina without finding red blood cells 14 days later. Often the sperm has been scanty and though this might be due to the length of time intervening between copulation and the examination of the vaginal smear, yet it is also possible that our bucks are less than normally fertile and some improvement in their diet might be desirable. We do not, however, wish to modify the diet of the does as the young they produce are richly enough, and yet not too richly, stored with vitamins to be suitable for vitamin tests, and if the diet were modified to increase fertility, the condition of the young for vitamin tests might be less good.

Seasonal variation in the reproduction of the rats.

For the last two years, we have taken about 30 young does per month for breeding on various diets, and there has certainly been a greater number of births in the summer months than in the winter, November, December and January being the worst. This might so easily have been due to seasonal differences in the fresh food of the diet and in the poorness of certain modifications of the diet, that we have not attached any great importance to it. We have, however, analysed the records of the rats that have been brought up entirely on C stock diet with dried milk, liver and watercress and we have found a drop in the number of litters born during November, December and January. Table VI summarises the figures on this point.

Table VI. *The seasonal variation in the reproduction of rats receiving the C stock diet with 20 % dried milk* plus *liver and watercress.*

		No. of does*	No. of litters weaned	No. of litters weaned per doe per month
August,	1930	10	8	0·80
September,	,,	22	14	0·64
October,	,,	30	11	0·37
November,	,,	37	9	0·24
December,	,,	63	8	0·13
January,	1931	83	17	0·20
February,	,,	95	28	0·29
March,	,,	128	35	0·27
April,	,,	158	54	0·34
May,	,,	171	49	0·29
June,	,,	172	69	0·40
July,	,,	155	46	0·30
August,	,,	153	57	0·37

* These numbers refer to does which were first mated at least 2 months before the month signified.

The seasonal variation is evident even if the numbers for the first 2 months are ignored as they probably should be. But there is no reason to suppose that this variation is due to any other cause than the diet; for the seasonal variation in the liver, watercress and dried milk may easily be great enough to account for the variation in the reproduction of the rats.

Rate of growth of the rats of this colony.

Composite growth curves have been drawn for the bucks and does of three generations respectively fed on C diet *plus* 20 % dried milk *plus* liver and watercress. The results are summarised in Table VII. It is evident that there is no tendency for the later generations to grow more slowly than the earlier ones. It has similarly been calculated that the rats of first litters do not grow more slowly than do rats of subsequent litters. It has also been determined that there is no seasonal variation in the average weights of bucks or does at 13 weeks of age.

Table VII. *Growth of rats fed on C diet* plus *20 % dried milk* plus *liver and watercress.*

	No. of animals	Average weight (g.) at 4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks	13 weeks	σ of weight at 13 weeks $\sigma=\sqrt{\frac{\Sigma d^2}{n-1}}$
BUCKS												
1st generation	70	46·0	62·3	84·2	104·9	122·7	139·9	153·6	165·2	178·0	186·7	26·196
2nd ,,	51	44·2	59·9	82·9	101·1	118·5	133·4	145·7	160·2	170·8	181·8	23·333
3rd ,,	5	45·7	62·0	84·0	104·2	125·4	145·2	154·0	165·2	179·4	206·7*	39·249
Weighted mean	126	45·3	61·3	83·7	103·4	121·1	137·5	150·4	163·1	175·0	185·3	26·0
DOES												
1st generation	241	42·6	56·4	71·9	84·8	96·8	107·0	115·8	124·1	130·5	131·1	13·55
2nd ,,	175	40·4	54·1	69·6	82·3	92·8	103·0	113·1	120·7	128·2	134·6	14·387
3rd ,,	31	42·9	58·3	75·4	89·7	101·9	110·8	120·0	127·9	134·7	139·8	13·111
Weighted mean	447	41·8	55·6	71·3	84·2	95·6	105·8	115·0	123·1	129·6	135·2	13·86

* Four only.

Suitability of the young of this colony for vitamin tests.

We have used the young of this colony for tests for vitamins A, D, B_1 and B_2. So far we have carried out no work on the other components of vitamin B.

1. *Vitamin A work.* While we were giving the stock rats liver twice a week, we found that the young weighing 40–50 g. when given our vitamin A-free diet grew far beyond the weight at which they should cease to grow (80–100 g.). We tried giving the vitamin A-free diet to certain does during the third week of lactation in order to reduce the reserves of vitamin A in the young and this succeeded with the litters which survived but was apparently too drastic when the A-free diet was given from the 14th day of lactation, for some of the litters died. When, however, the diet was given from the 17th day of lactation, the young never died. We, therefore, now give beef to all the stock rats three times and liver only once in a fortnight. We choose rats weighing about 30 g. for vitamin A tests and we find that these cease to grow at about 80–100 g. weight. Occasional bucks grow above 120 g. and does above 110 g. but these we discard.

2. *Vitamin B tests.* Young rats from this colony are suitable for vitamin B tests (B_1 and B_2) at 55–60 g. weight.

3. *Vitamin D tests.* For prophylactic tests we use rats weighing about 40 g. For curative tests we use rats weighing about 60 g. at the beginning of the preparatory period. The room in which the tests are carried out faces east and has yellow blinds always drawn over the windows. In Fig. 1 we have indicated the average degree of rickets produced by the rachitogenic diet in litters of rats from does fed from weaning on the C stock with 20 % dried milk, liver and watercress. It is evident that we are getting a more and more nearly uniform, severe degree of rickets. There is no obvious seasonal variation in the degree of rickets developed. Some workers attribute variations in the degrees of rickets produced to a variation in the amount of vitamin D in their

stocks of yellow maize. We have no information on this point as we buy 1 cwt. of whole yellow maize per week (using it in the stock diet as well as in the rachitogenic diet) and grind it for use as required. Variations in such small quantities would not be noticeable. It seems to us that the fluctuations in severity of rickets developed in our rats can most probably be ascribed to variations in the vitamin D content of the dried milk of the stock diet. From May to July, 1930, we used a sample which had been specially dried for us by the Glaxo Co. in the previous February. From August to November we used a sample which the same firm dried for us in late July. We then used a dried milk, bought in the open market, until March, 1931, when we again had a sample of milk specially dried for us. We then used this sample and the one bought monthly in the open market during alternate weeks until October 1931,

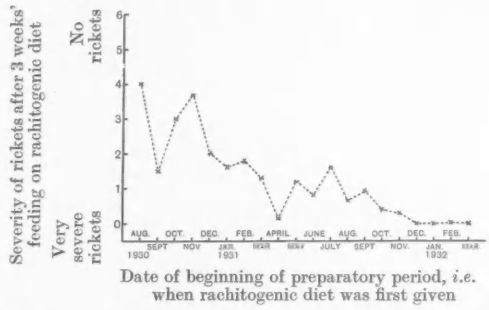

Fig. 1. To show the degree of rickets developed in litters of rats from does fed on C diet + 20 % dried milk + liver and watercress, measured by the method described by Dyer [1931].

after which we used the one bought in the open market continuously. It seems to us, that having once reduced the vitamin D reserves of our colony, they can be kept low by the use of winter dried milk in the stock diet. It would appear to be safe to rely on the low vitamin D content of dried milk bought in the open market in winter, if it were known to be prepared from fresh English milk. New Zealand dried milk might be richer in this factor but we have no information on this point. Our use of English dried milk bought in the open market in the summer seems to raise the vitamin D reserves of the young litters even when it is used in alternate weeks only, dried winter milk being used in the other weeks. Even so, the degree of rickets is still severe, and it may be advantageous for the colony to have a limited supply of dried summer milk rather than dried winter milk continuously.

Summary.

Evidence is given that a diet found to be suitable for a colony of rats in one laboratory was not satisfactory in this laboratory where it was made from the same recipe but necessarily of materials from different sources. The

deficiency was not determined though it was shown to be none of the recognised vitamins.

A second diet recommended by other workers was also found to be unsatisfactory in this laboratory though the nature of the deficiency was not as fully investigated as that of the first diet.

A fresh diet was devised which, with certain modifications, has proved to be satisfactory for at least two generations of rats. Furthermore, evidence is given that the young of the rats fed on this diet are suitable for tests for vitamins A, B_1, B_2 and D.

Reproduction on this diet shows a definite seasonal variation which may be accounted for by the variation in certain of the constituents of the diet (fresh liver, watercress and dried milk). There was, however, no seasonal variation in the growth of the young rats on this diet. Fluctuations in the degree of rickets produced in the young under certain conditions appear to be correlated with the source of the dried milk of the stock diet, and, with this controlled, the fluctuations are not serious, and gradually disappear.

The writers would express their gratitude to Mr W. Bedford for his generous supplies of watercress for the colony during many years.

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LXXXVI. VARIATION IN GROWTH RESPONSE OF RATS IN VITAMIN A TESTS COMPARED WITH THE VARIATION IN RATS GROWING NORMALLY.

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Routine tests for vitamin A in foodstuffs carried out in these laboratories are based on the power of the substance under test to restore growth in animals which have ceased to grow on a diet considered deficient only in vitamin A. The test is made in direct comparison either with some other foodstuff convenient for comparison, or with a particular sample of cod-liver oil which we have for some time used as a standard of reference [Morgan and Coward, 1931; Coward, Dyer, Morton and Gaddum, 1931]. The method of comparison is as follows. About 10 rats which have become steady in weight on a diet deficient in vitamin A are all given equal daily doses of the substance under test for 5 weeks, and about 10 similar rats are all given equal daily doses of the substance with which comparison is being made. The average increase in weight for the period is determined for each group, and the comparison between the vitamin A contents of the two doses is drawn by reference to the curves described by Coward *et al.* [1930]. That is, the relative amounts of the standard cod-liver oil which would have brought about these mean increases are determined, and from the figures so obtained are calculated the relative potencies of the two substances compared. Greater accuracy is obtained by comparing bucks with bucks and does with does according to the curves described by the same writers [1931].

The problem then presented itself of how far small differences in average response were significant, and calculations were made for each test from the formula

$$\frac{x_1 - x_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}},$$

in which x_1 and x_2 are the mean increases in weight of the two groups of rats in 5 weeks, and ϵ_1 and ϵ_2 are the standard errors of the two groups;

$$\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

It was tentatively accepted that if the value so obtained were greater than 2, the difference was significant.

This, however, appeared to be unsatisfactory for sometimes ϵ was small and sometimes large. It became evident that it would be sounder to obtain the average standard deviation and apply it to all tests. Dr J. H. Gaddum, when consulted on this matter, suggested the finding of the average (weighted) value of $\sigma^2\left[=\frac{\Sigma d^2}{n-1}\right]$ for as many tests as possible and the use of it in comparisons in the formula

$$\frac{x_1-x_2}{\sqrt{\frac{\sigma^2}{n_1}+\frac{\sigma^2}{n_2}}}\left[=\frac{x_1-x_2}{\sigma\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}\right],$$

which would have a value greater than 2 if x_1-x_2 were significant. This has been done and also, for comparison, the standard deviation of the mean increases in weight in 5 weeks of rats from our normal colony has been calculated.

Table I. *The standard deviation of 5 weeks' increase in weight of normal rats according to the age and weight at the beginning of the period.*

Standard deviation (S.D.) calculated as $\sqrt{\frac{\Sigma d^2}{n-1}}$; all weights in g.

		Periods of 5 weeks beginning at the				
	No. of rats	4th week of life	5th week of life	6th week of life	7th week of life	8th week of life
Bucks—1st generation	54					
Av. initial weight		46·7	61·8	84·0	104·3	121·5
Mean increase		92·2	91·1	81·7	73·9	65·3
S.D. of mean increase		17·31	17·46	15·89	15·97	16·71
S.D. as % of mean increase		18·8	19·2	19·5	21·6	25·6
Bucks—2nd generation	41					
Av. initial weight		44·2	60·6	84·9	103·1	121·3
Mean increase		92·5	88·0	78·7	71·0	64·0
S.D. of mean increase		18·99	20·15	20·49	16·18	13·556
S.D. as % of mean increase		20·6	22·9	26·0	22·8	21·2
Bucks—3rd generation	5					
Av. initial weight		—	61·8	84·0	104·2	—
Mean increase		—	92·2	81·2	75·2	—
S.D. of mean increase		—	23·68	23·82	27·88	—
S.D. as % of mean increase		—	25·7	29·3	37·1	—
Does—1st generation	204					
Av. initial weight		42·4	55·9	71·7	84·4	95·9
Mean increase		63·6	59·2	52·0	45·9	40·1
S.D. of mean increase		11·94	11·62	10·83	9·59	8·91
S.D. as % of mean increase		18·8	19·6	20·8	20·1	22·2
Does—2nd generation	143					
Av. initial weight		40·3	54·4	70·2	83·4	93·7
Mean increase		63·6	59·1	50·8	44·9	41·0
S.D. of mean increase		13·38	12·51	12·15	11·12	10·35
S.D. as % of mean increase		21·0	21·2	23·9	24·8	25·2
Does—3rd generation	22					
Av. initial weight		41·9	58·6	76·5	90·9	102·6
Mean increase		69·7	62·9	52·7	44·7	37·5
S.D. of mean increase		11·26	9·42	8·41	6·64	6·66
S.D. as % of mean increase		16·2	15·0	15·95	14·85	17·8

The standard deviation (σ) of a single determination of the increase in weight of normal rats in 5 weeks.

In case the standard deviation should depend on the initial weight of the group of rats, we calculated this function for periods beginning at the 4th, 5th, 6th, 7th and 8th weeks respectively, of the rats' lives. The results of this calculation are collected in Table I.

It is interesting to note that there is a tendency in the standard deviation of the mean increase to decrease as the initial weight of the group increases, but when expressed as a percentage of the mean increase, this figure increases as the initial weight of the group increases. Perhaps the figures that are most useful for comparison with the results in the vitamin A tests are the standard deviations of the increases of those rats which were about the same weight at the beginning of some selected 5 weeks' period as the rats at the beginning of the 5 weeks' tests. That is $\sigma = 17$ (approx.) for bucks (the period 6–11 weeks) and 10 (approx.) for does (the period 7–12 weeks). (These numbers also happen to agree very well with the averages of all the standard deviations of the normal bucks and does respectively.) Thus the normal standard deviation of the mean increase in weight during 5 weeks' growth may be accepted as about 17 for bucks (initial weight, 84·5 g.) and about 10 for does (initial weight, 81·4 g.).

The standard deviation (σ) of a single determination of the increase in weight of rats in 5 weeks in vitamin A tests.

The results of 108 tests for vitamin A are summarised in Table II. They are all comparable in so far as they have all been carried out under similar conditions of basal diet, housing, *etc.* In each test all the animals received

Table II. *The standard deviation in the increase in weight of 108 groups of experimental rats (bucks and does calculated separately) which have become steady in weight on a diet deficient in vitamin A and have then been given a daily dose for 5 weeks of some substance to be tested for vitamin A.*

No. of test	No. of rats ♂	No. of rats ♀	Mean increase g. ♂	Mean increase g. ♀	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♂	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♀
1	7	7	−19·3	−4·0	13·13	11·58
2	17	7	12·5	13·3	9·70	5·45
3	10	17	18·8	16·1	14·48	15·79
4	19	11	30·6	22·6	10·35	10·02
5	18	12	55·7	36·9	11·67	10·93
6	15	16	53·0	37·2	14·38	9·21
7	10	9	4·2	11·8	10·72	12·75
8	4	5	35·7	24·6	14·80	8·12
9	7	10	−0·9	−0·8	8·07	12·17
10	6	13	27·7	20·2	12·44	5·27
11	13	4	61·4	37·2	19·09	13·67
12	7	11	54·9	36·2	19·67	12·12
13	8	12	2·5	6·3	8·16	8·17
14	8	11	15·0	17·0	14·71	15·22
15	10	16	19·2	9·4	12·66	15·61

Table II (*cont.*)

No. of test	No. of rats ♂	No. of rats ♀	Mean increase g. ♂	Mean increase g. ♀	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♂	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♀
16	10	11	18·0	14·9	11·75	8·91
17	10	11	1·7	9·8	7·41	17·27
18	8	12	40·4	27·2	19·59	9·15
19	14	6	42·4	30·3	10·05	17·22
20	8	8	18·4	18·5	12·98	4·72
21	10	9	9·7	7·0	12·76	12·62
22	10	12	14·6	15·3	12·02	7·28
23	2	8	31·5	22·8	0·71	9·32
24	7	7	13·7	12·0	10·95	8·26
25	6	4	−4·0	−2·0	19·13	8·04
26	5	5	1·8	6·2	6·83	15·77
27	7	3	16·4	11·0	16·64	3·46
28	4	5	27·0	22·6	12·68	6·60
29	5	3	−0·4	6·0	16·29	5·29
30	5	5	18·2	24·8	14·53	6·94
31	6	6	7·8	6·7	11·99	11·70
32	7	7	16·1	13·7	14·46	11·73
33	6	6	9·3	14·5	13·62	16·71
34	9	4	11·4	9·2	18·19	19·97
35	5	5	32·4	30·0	8·97	7·18
36	4	8	2·2	9·9	32·29	12·34
37	6	5	35·3	37·8	32·62	8·98
38	4	6	37·2	25·5	18·47	6·37
39	3	6	27·0	18·0	21·63	7·40
40	6	4	15·8	19·2	20·82	8·93
41	4	5	−20·2	5·6	5·63	14·02
42	4	5	44·2	36·8	16·52	3·77
43	5	6	13·4	13·7	16·30	17·95
44	4	6	46·0	37·7	6·48	17·16
45	4	6	−2·5	−2·8	7·05	9·83
46	8	8	−3·6	4·5	19·73	8·85
47	5	5	18·0	11·4	13·62	7·60
48	4	5	27·7	18·4	8·42	7·37
49	6	5	−0·3	0·6	7·54	9·08
50	5	5	24·8	28·2	18·67	4·09
51	4	3	47·0	27·3	12·30	11·73
52	6	4	24·8	13·2	22·62	7·42
53	3	3	45·0	20·0	10·58	8·54
54	5	4	34·6	26·5	2·88	5·07
55	8	2	25·0	30·5	18·14	13·45
56	5	5	32·8	35·0	3·96	6·36
57	4	6	42·2	27·7	21·12	6·07
58	3	5	14·7	11·4	6·04	14·16
59	5	5	27·0	29·4	13·10	12·12
60	4	6	25·7	26·5	10·78	8·12
61	8	12	19·1	15·8	25·28	13·96
62	5	4	29·6	30·7	19·77	4·93
63	5	5	41·4	30·4	12·83	3·87
64	6	5	66·8	39·0	10·54	11·49
65	5	5	30·8	35·0	13·06	6·33
66	4	6	10·7	16·0	13·60	17·02
67	5	4	16·0	13·5	23·37	14·40
68	7	3	7·0	10·0	10·45	13·75
69	3	3	26·3	32·7	2·12	17·01
70	3	6	26·3	16·0	5·86	15·82
71	2	5	15·5	6·4	10·63	12·82
72	4	6	16·0	12·5	12·68	13·10
73	7	4	−0·1	12·2	19·39	16·28
74	4	6	16·5	7·5	11·75	8·16
75	4	6	32·0	17·0	8·08	4·90

Table II (*cont.*)

No. of test	No. of rats ♂	No. of rats ♀	Mean increase g. ♂	Mean increase g. ♀	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♂	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ ♂
76	4	6	28·5	22·7	10·30	3·22
77	5	5	46·8	34·2	14·77	5·40
78	2	7	34·5	36·7	55·86	11·58
79	6	4	3·0	16·7	18·41	12·58
80	4	7	7·0	21·3	11·78	16·19
81	7	3	34·0	14·7	18·33	9·82
82	2	7	27·5	25·1	20·50	10·73
83	6	4	45·8	29·5	16·67	7·79
84	5	5	25·0	21·8	13·58	10·75
85	3	5	15·3	20·4	10·02	4·30
86	5	5	44·6	21·6	17·13	4·00
87	4	6	12·7	30·3	11·09	6·63
88	4	4	38·5	23·5	10·52	7·57
89	4	5	38·7	33·4	16·28	8·57
90	3	7	41·0	30·6	2·00	11·69
91	3	5	34·3	17·4	18·15	6·32
92	4	5	34·5	20·4	14·25	5·29
93	4	6	83·0	50·2	12·11	5·31
94	4	5	77·0	45·4	5·26	11·85
95	5	5	7·0	9·0	6·67	7·97
96	5	5	31·4	20·6	3·74	6·82
97	5	4	−4·8	0·7	19·83	3·70
98	6	4	9·3	8·5	17·49	7·53
99	2	8	51·5	30·6	13·45	10·52
100	2	8	49·5	36·6	0·71	12·09
101	4	6	41·0	14·5	3·56	7·86
102	4	3	48·5	34·7	14·26	11·02
103	5	3	17·0	28·7	7·60	4·36
104	5	4	27·4	29·7	11·47	16·40
105	7	2	19·9	13·5	15·69	0·71
106	4	6	56·2	44·5	19·62	10·72
107	2	6	30·0	18·8	22·63	13·62
108	6	2	29·0	3·5	8·85	23·33

Total number of tests = 108
" bucks = 635 } = 1307 rats
" does = 672 }

Average initial weight of bucks = 86·9 g.
" " does = 83·6 g.

$$\text{Average variance} = \sigma^2 = \frac{\Sigma d^2}{N - m}$$

where N = the total number of observations and m = the number of means (*i.e.* N = the number of rats, m = the number of tests);

for bucks = 216·74
for does = 121·44.

Therefore the standard deviation of a single determination

for bucks = 14·72
for does = 11·02.

the same daily dose. The substances tested and their doses are immaterial in the present investigation and are therefore not included. The number of rats (bucks and does respectively), average increase in weight in 5 weeks and the

standard deviation for a single determination $\left[\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}\right]$ of the increase in weight, have been tabulated for each of the tests.

The average initial weights of the groups of bucks and does respectively are indicative of the weights at which the rats of our colony cease to grow when given a diet deficient in vitamin A. Occasional rats have not ceased to grow at a weight of 120 g. (bucks) or 110 g. (does) and are, therefore, not used for tests. It is interesting to note that the average weight at which 635 bucks ceased to grow on the vitamin A-free diet was 86·9 g. and that at which 672 does ceased to grow was 83·6 g. which is not much less than that of the bucks. This would appear to indicate that the reserves of vitamin A of the young bucks when first given the vitamin A-free diet are not appreciably greater than those of the young does.

The mean increases in weight of the bucks and does in 5 weeks necessarily varied greatly as the doses of substances tested varied greatly in their vitamin A contents. The final average for the whole number of 108 tests is not of interest except perhaps in supplying an average figure when correlating different standard deviations with different mean increases (see later part of paper).

The standard deviations of single determinations show a wide range among the various tests. The average standard deviation is 14·72 for the bucks and 11·02 for the does.

This result means that about 1 of every 3 bucks used in a test may be expected to give an increase in weight of 14·72 g. more or less than the average increase in weight. Similarly 1 of every 3 does may be expected to give an increase in weight of 11·02 g. more or less than the average increase. This would appear to show that it is more accurate to use does for these tests than bucks, but actually this is not so, for the curve of response to doses of vitamin A is steeper for bucks than for does, which more than compensates in accuracy for the greater standard deviation (see later part of paper).

Variation in the standard deviation.

Before the figures 14·72 (bucks) and 11·02 (does) were accepted as applicable to all tests, an analysis was made to see whether the value of σ varied (*a*) according to the average initial weight of the group, *i.e.* the weight at which the rats became steady in weight and the 5 weeks' test period was begun, and (*b*) according to the mean increase in weight of the group. The result of the first analysis is shown in Table III. The tests have been grouped according to the average initial weights of the rats and the different values of the standard deviations for the various tests in each group have been averaged. The numbers of tests in each group are also recorded. It may be seen that there is no correlation between standard deviation and average initial weight of the group of rats on test. Similarly, in Table IV, in which the tests have been grouped according to the mean increase in weight of each group, it may be

Table III.

Average values of σ when the average initial weights of the groups are:

	Up to 75 g.	75–80 g.	80–85 g.	85–90 g.	90–95 g.	95–100 g.	>95 g.	>100 g.
♂	14·24	14·03	15·19	18·07	14·51			16·36
♀	11·98	11·70	10·94	11·28	9·86		7·70	
No. of tests								
♂	26	18	19	18	15			12
♀	22	17	20	20	18		11	

Table IV.

Average values of σ when the mean increases of the groups are:

	Up to 10 g.	10–20 g.	20–30 g.	More than 30 g.
♂	14·63	14·58	15·26	16·44
♀	12·57	11·43	8·60	10·82
No. of tests				
♂	23	26	18	41
♀	23	32	27	26

seen that the standard deviation does not vary according to the mean increase in weight of the groups.

Hence it would appear to be safe to adopt 14·72 for bucks and 11·02 for does as the standard deviation of a single determination of the increase in weight in 5 weeks whatever the average initial weight of the group and whatever the mean increase in weight in 5 weeks.

This raises the question of how far the accuracy of the final result of a test is affected by the general acceptance of these standard deviations for all mean increases in weight. In particular, how far does the range of results (2 out of 3 times) differ in a test where the mean increase is, say, 0, from the range in one where the mean increase is, say, 30 g.? This has been calculated in Table V for mean increases of 0, 10, 20, 30 g. respectively for both bucks and does. From the curves relating mean increase in a group of rats to the dose of vitamin A given [Coward, Key, Dyer and Morgan, 1930, 1931], the

Table V. *To show that the degree of accuracy of the test is the same, however the mean increases of the groups may vary.*

Mean increase, say: ♂ g.	♀ g.	Equiv. dose of oil A ♂ mg.	♀ mg.	Range of increase ♂ $\sigma=14{\cdot}72$ g.	♀ $\sigma=11{\cdot}02$ g.	Range of equiv. dose of oil A ♂ mg.	♀ mg.	Percentage range of equiv. doses ♂	♀
0	0	0·5962	0·3528	+14·72	+11·02	1·1695	0·8904	196·2	252·4
				−14·72	−11·02	0·3039	0·1397	51·0	39·6
10	10	0·9423	0·8173	+24·72	+21·02	1·8484	2·0635	196·2	252·5
				− 4·72	− 1·02	0·4803	0·3237	51·0	39·6
20	20	1·4894	1·8940	+34·72	+31·02	2·9214	4·7818	196·1	252·5
				+ 5·28	+ 8·98	0·7591	0·7502	51·0	39·6
30	30	2·3540	4·3883	+44·72	+41·02	4·6175	11·0790	196·1	252·5
				+15·28	+18·98	1·1998	1·7382	51·0	39·6

dose of a cod-liver oil that would have brought about each of these mean increases has been calculated. Similarly, the doses corresponding to mean increases in weight of $0 \pm 14{\cdot}72$ g., $10 \pm 14{\cdot}72$ g., $20 \pm 14{\cdot}72$ g., $30 \pm 14{\cdot}72$ g. (bucks) and also $0 \pm 11{\cdot}02$ g., $10 \pm 11{\cdot}02$ g., $20 \pm 11{\cdot}02$ g., $30 \pm 11{\cdot}02$ g. (does) have been calculated. Then each of these doses has been further calculated as a percentage of the dose corresponding to the mean increase. It may be seen in the last columns of Table V that the range of doses is about 50–200 % of the mean according to the bucks, and about 40–250 % according to the does, no matter what the mean increase in weight of the group. Thus it is shown that the degree of accuracy obtained by the use of the same values of the standard deviation is the same whatever the mean increase in weight from which the calculation is made. The fact that the does give a wider range than the bucks, *i.e.* a less accurate result in spite of their having a smaller standard deviation, is due to the growth curve of the does being less steep than that of the bucks.

The standard error (ϵ) of the mean.

The foregoing determinations have dealt with the standard deviation of a single determination. It is necessary to find how far the accuracy of an average result is affected by the number of animals in the group used for the test. In other words, the standard deviation of the mean (*i.e.* $\sigma/\sqrt{n}$) with different values for n must be determined. This is shown in some detail in Tables VI and VII. In Table VI a mean increase of 0 g. for bucks and does

Table VI. *To show that when 10 rats (bucks or does, but not a mixture) are used in a test, the vitamin value of the dose will be within 25 % above and 21 % below (according to the bucks), and within 35 % above and 25 % below (according to the does) the true value, two out of three times of performing the test. Similarly the result will be within 56 % above and 37 % below (according to the bucks), and within 80 % above and 44 % below (according to the does) the true value, 21 out of 22 times of performing the test.*

Standard error of the mean $\left(\epsilon = \frac{\sigma}{\sqrt{n}}\right)$.

When the number of rats is 10, $\epsilon = 4{\cdot}66$ (bucks) and $\epsilon = 3{\cdot}48$ (does).

Mean increase ♂ g.	Mean increase ♀ g.	Equiv. dose of oil A ♂ mg.	Equiv. dose of oil A ♀ mg.	Range of mean increase when $n=10$ ♂ g.	Range of mean increase when $n=10$ ♀ g.	Range of equiv. dose of oil A ♂ mg.	Range of equiv. dose of oil A ♀ mg.	Percentage range of equiv. doses ♂	Percentage range of equiv. doses ♀
				(*a*) $\pm\epsilon$ where $\epsilon = \frac{\sigma}{\sqrt{n}}$.					
0	0	0·5962	0·3528	+4·66 −4·66	+3·48 −3·48	0·7379 0·4817	0·4726 0·2633	123·8 80·8	134·0 74·6
				(*b*) $\pm 2\epsilon$.					
				+9·32 −9·32	+6·96 −6·96	0·9129 0·3891	0·6331 0·1965	153·1 65·3	179·5 55·7

Table VII. *The degrees of accuracy obtainable with different numbers of rats.*

No. of rats	Mean increase ♂ g.	Mean increase ♀ g.	Equiv. dose of oil A ♂ mg.	Equiv. dose of oil A ♀ mg.	Value of $\epsilon = \frac{\sigma}{\sqrt{n}}$ ♂ g.	Value of $\epsilon = \frac{\sigma}{\sqrt{n}}$ ♀ g.	Value of 2ϵ ♂ g.	Value of 2ϵ ♀ g.	Range of equiv. doses of oil A (*i.e.* mean $\pm 2\epsilon$) ♂ mg.	Range of equiv. doses of oil A (*i.e.* mean $\pm 2\epsilon$) ♀ mg.	Percentage range of equiv. doses ♂	Percentage range of equiv. doses ♀
2	20	20	1·4894	1·8940	10·48	7·93	20·96	15·86	3·8877 0·5705	7·1812 0·4994	261·0 38·3	379·1 26·4
3	—	—	—	—	8·50	6·36	17·00	12·72	3·2427 0·6839	5·5158 0·6503	217·7 45·9	291·2 34·3
5	—	—	—	—	6·58	4·93	13·16	9·86	2·7202 0·8153	4·3371 0·8270	182·6 54·7	229·0 43·7
10	—	—	—	—	4·65	3·48	9·30	6·96	2·2793 0·9734	3·3995 1·0554	153·0 65·4	179·5 55·7
15	—	—	—	—	3·80	2·84	7·60	5·68	2·1086 1·0517	3·0549 1·1752	141·6 70·6	161·3 62·0
20	—	—	—	—	3·29	2·46	6·58	4·92	2·0128 1·1018	2·8635 1·2526	135·2 74·0	151·2 66·1
25	—	—	—	—	2·94	2·20	5·88	4·40	1·9494 1·1384	2·7416 1·3086	130·9 76·4	144·8 69·1
36	—		—	—	2·45	1·84	4·90	3·68	1·8638 1·1899	2·5805 1·3785	125·1 79·9	136·2 72·8

has been selected (*i.e.* maintenance of weight). The doses of the standard oil which would just maintain weight in bucks and does respectively have been determined from the curves already described in previous papers. The ranges of the mean increases in weight when the number of animals in the group is 10 have been calculated from the formula $\epsilon = (\sigma/\sqrt{10})$. The range of doses of the standard oil corresponding to the range of mean increases has been calculated from the curves and expressed as percentages of the actual doses in the last column. This shows that 2 out of 3 tests with 10 animals in each test will give a value within 24 % above and 20 % below the true value according to the bucks, and within 34 % above and 26 % below, according to the does.

Similarly values have been calculated for ranges of $\pm 2\epsilon$, and from these it is seen that in 21 out of 22 tests with 10 animals in each test the values found will lie within 54 % above and 35 % below the true value according to the bucks, and within 80 % above and 45 % below according to the does.

The degree of accuracy obtainable with different numbers of rats.

In Table VII are calculated in the same way the degrees of accuracy obtainable from different numbers of animals. They are most easily estimated by reference to Fig. 1 in which the percentage range of accuracy has been plotted against the number of animals used in the test. The uppermost and lowest curves represent the results obtainable with does, the middle ones represent those obtainable with bucks. It is easily seen that an increase in the number of animals gives a greater degree of accuracy but it is evident that the higher numbers (say 20–36) would bring about so small an increase in accuracy over that obtained with, say, 10 rats, that it would be disproportionate to the extra work involved in handling 20–36 rats rather than 10. To obtain a result 21 out of 22 times within 50 % above and 33 % below, 11 bucks

or 21 does would be required. To obtain a result within 60 % above and 38 % below, 8 bucks or 15 does would be required. Smaller numbers of animals than these would give disproportionately greater degrees of inaccuracy, and it would appear to be a practical outcome of the work to suggest the use of 8 bucks or 15 does for a vitamin A test. This would give a 21/22 probability of being within 60 % above and 38 % below the true value and a 2/3 probability of being within 27 % above and 21 % below the true value.

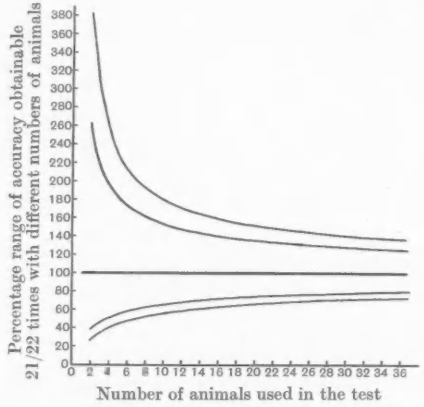

Fig. 1. To show the range of values, calculated as a percentage of the true value, obtainable with different numbers of rats, the range being given as a 21/22 probability of the value lying within $\pm 2\epsilon$ where ϵ is the standard error of the determination made with the stated number of animals.

The ratio of bucks to does giving a particular degree of accuracy is shown by the curves to be the same, 1:2 (approx.) at all percentages of accuracy. This is determined partly by the different standard deviations of the single determination, 14·72 for bucks, 11·02 for does, and partly by the fact that the slope of the curve of growth for bucks is greater than that for does.

The standard deviation of the logarithm of the result (λ).

The standard deviation of the logarithm of the result is obtained by dividing σ by the slope of the line connecting the response with the log (to base 10) of the dose [Coward, Dyer, Morton and Gaddum, 1931]. Since the curve relating the response of bucks to the log of the dose is $y = 11{\cdot}3 + 50{\cdot}3x$ (where x = the log of the dose) and that for does is $y = 12{\cdot}4 + 27{\cdot}4x$, then the standard deviation of the logarithm of the result is

$$\frac{14{\cdot}72}{50{\cdot}3} = 0{\cdot}293 \text{ for bucks } (\lambda_1),$$

and

$$\frac{11{\cdot}02}{27{\cdot}4} = 0{\cdot}402 \text{ for does } (\lambda_2).$$

The error of the test is then $\frac{\lambda_1}{\sqrt{n}}$ for the bucks and $\frac{\lambda_2}{\sqrt{n}}$ for does. (λ^2 has been termed the "logarithmic variance" of the test.)

From this λ_1^2 is seen to be 0·085849 and λ_2^2 is seen to be 0·161604.

That is λ_1^2 is about half of λ_2^2, which agrees with the previous finding that about twice as many does as bucks are required for any given degree of accuracy.

Significant differences in mean increases in weight of two groups of rats.

To determine significant differences in mean increases in weight of any two groups of rats, the formula

$$\frac{x_1 - x_2}{\sigma\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

has been used.

x_1 and x_2 = mean increases of the two groups respectively,
σ = 14·72 (bucks),
= 11·02 (does),
n_1 and n_2 = the number of animals in the two groups respectively.

If the value so found is equal to or greater than 2, the difference may be considered significant. Obviously the greater the value of n, the smaller need be the value of $x_1 - x_2$ for the difference to be considered significant. In Table VIII (column 2) values of $x_1 - x_2$ have been worked out for various values of n (n_1 and n_2 being made equal in each calculation for the sake of simplicity). Further, by taking a mean increase of 10 g. for one test and 10 *plus* each value of $x_1 - x_2$ so found for the other (column 3), and determining the dose of the standard oil (column 4) which would bring about these mean increases, there have been determined the percentage differences (column 5) in the vitamin values of the doses of substance tested, which were indicated by the various values of $x_1 - x_2$. Thus, if only 7 bucks were used for testing each of two samples, the difference between the mean increases of the groups would have to be at least 15·75 g. to show that the dose of one substance tested differed significantly from the other and then it would appear to have 204·2 % of the value of the other dose. Similarly the use of 25 bucks on each dose would have to give a difference between the mean increases of at least 8·33 g. to allow the conclusion that the dose of one substance had 145·3 % the value of the other. Figures for the does have also been calculated.

Dr J. H. Gaddum has suggested that when a comparison between two substances is made by using two groups of animals, each of which contains both bucks and does, the relative potency of the substances as indicated by the bucks and the relative potency as indicated by the does may best be averaged by the use of the formula:

$$\frac{\frac{n_1 x_1}{\lambda_1^2} + \frac{n_2 x_2}{\lambda_2^2}}{\frac{n_1}{\lambda_1^2} + \frac{n_2}{\lambda_2^2}},$$

where n_1 = the total number of bucks used in the comparison; n_2 = the total number of does used in the comparison; x_1 = the log of the ratio of the potency of the two substances according to the bucks; x_2 = the log of the ratio of the potency of the two substances according to the does; λ_1^2 = the logarithmic variance of the bucks; λ_2^2 = the logarithmic variance of the does.

Table VIII. *To show (a) the least value that the difference $(x_1 - x_2)$ between two mean increases must have in order to be considered significant when different numbers of animals are used for comparisons, and (b) the corresponding differences in vitamin A value which are indicated by these significant differences in mean increases.*

	No. of animals (1)	Least value of $x_1 - x_2$ to be significant (2) g.	Mean increases (say) (3) g.	Doses of standard oil which would have brought about these increases (4) mg.	Value of richer substance stated as a percentage of value of poorer substance (5)
♂	3	24·02	10	0·95	297·9
			34·02	2·83	
	5	18·61	10	0·95	232·6
			28·61	2·21	
	7	15·75	10	0·95	204·2
			25·75	1·94	
	9	13·87	10	0·95	188·0
			23·87	1·78	
	12	12·01	10	0·95	172·6
			22·01	1·64	
	16	10·42	10	0·95	160·0
			20·42	1·52	
	25	8·33	10	0·95	145·3
			18·33	1·38	
♀	3	17·98	10	0·82	451·2
			27·98	3·70	
	5	13·93	10	0·82	322·1
			23·93	2·64	
	7	11·79	10	0·82	268·3
			21·79	2·20	
	9	10·38	10	0·82	239·0
			20·38	1·96	
	12	8·99	10	0·82	212·2
			18·99	1·74	
	16	7·80	10	0·82	191·5
			17·80	1·57	
	25	6·23	10	0·82	168·3
			16·23	1·38	

If we accept the finding that twice as many does as bucks are required to obtain the same degree of accuracy, then the formula reduces to $\frac{2n_1x_1 + n_2x_2}{2n_1 + n_2}$ of which the logarithmic variance is given by the formula $\frac{1}{\lambda^2} = \frac{n_1}{\lambda_1^2} + \frac{n_2}{\lambda_2^2}$.

SUMMARY.

The results of 108 tests (involving 1307 rats) for vitamin A carried out in this laboratory under similar conditions of basal diet, housing *etc.* have been examined with a view to determining the degree of accuracy obtainable with reasonably small numbers of animals in general tests for vitamin A. The growth of 469 normal rats has been examined for comparison.

The standard deviation $\left(\sigma = \frac{\Sigma d^2}{n-1}\right)$ of a single determination of the increase in weight of rats (bucks or does) in a 5 weeks' growth test for vitamin A is not different from that of the normal increase in weight of rats (bucks or does) for the same period and beginning at the same average weight.

The standard deviation ($\sigma = 14{\cdot}72$ for bucks and $11{\cdot}02$ for does) of the experimental animals is the same whatever the mean increase in the test may be and whatever the average initial weight of the groups may be.

The standard deviation allows the same percentage range of accuracy whether the mean increase in weight and corresponding dose of standard oil are low or high. This has been shown by evaluating the vitamin A content of a dose of test substance in terms of the standard oil by means of the curves described by Coward, Key, Dyer and Morgan.

The degrees of accuracy obtainable with different numbers of rats (bucks or does) have been calculated. To get equal degrees of accuracy about twice as many does as bucks must be used in a test; this is due to the steeper curve relating the growth of bucks to the dose, which more than compensates for their greater standard deviation.

The difference between mean increases in weight of two groups of animals which must be obtained in order that the results may be considered significantly different has been calculated for different numbers of animals in the groups compared. The corresponding percentage differences in vitamin value of the doses compared have also been calculated.

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LXXXVII. THE VITAMIN C CONTENT OF SHEEP-LIVER; WITH OBSERVATIONS ON THE EFFECTS OF FREEZING AND STORAGE.

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(*Received April 27th, 1932.*)

Previous workers have shown that liver may contain an appreciable amount of vitamin C; *e.g.* rat-liver [Parsons, 1920]; chicken-liver [Hart, Steenbock, Lepkovsky and Halpin, 1925]; calf-liver [Thurston, Eckles and Palmer, 1929]; but no investigation of the antiscorbutic activity of sheep-liver has hitherto been carried out. The results of the experiments described below show this to be considerable, and in view of the fact that large quantities of livers are imported into this country in the frozen state from overseas it was of interest to investigate the effects of freezing and of storage on the vitamin C content. In the course of the work conclusions have been reached as to the influence of freezing in inactivating the vitamin.

1. Vitamin C content of sheep-liver.

Preparation of material. It proved impracticable to feed the liver directly to guinea-pigs receiving a scorbutic, but otherwise complete diet, since severe digestive trouble and loss of appetite rapidly ensued and the liver intake could not be controlled. Aqueous extracts of the liver did not have this effect, and were employed instead, the preparation being carried out as follows. Portions of sheep-liver were minced and passed through a fine wire sieve. The finely divided liver was then weighed into a bottle, and an equal weight of water added. The mixture was next shaken for 20 minutes in a mechanical shaker, the air having first been replaced by carbon dioxide to prevent possible inactivation of vitamin C by oxidation. The mixture was freed from particles of connective tissue by filtration through flannel and the filtrate centrifuged. The supernatant fluid was then decanted off and fed quantitatively from a pipette to guinea-pigs immediately after preparation.

The maximum liver equivalent of the aqueous extract was calculated as follows. To 100 g. of liver were added 100 cc. of water. Since the water content of sheep-liver is 71·3 % [Plimmer, quoted by McKillop, 1930] the total water content of the mixture is 171·3 g. Assuming that the water-soluble vitamin C is dispersed in the aqueous phase and not adsorbed on the solid surfaces on centrifuging, 1 cc. of the supernatant fluid corresponds with $\frac{100}{171{\cdot}3}$ g. liver and therefore 10 cc. of the supernatant fluid are equivalent in vitamin C content to 5·77 g. liver.

Feeding experiments. Two different methods were employed to measure the antiscorbutic activity of the aqueous extract of sheep-liver.

(1) Curative action on guinea-pigs of graded doses of the extract in comparison with that of cabbage.

(2) Determination of the smallest amount of extract added daily to the basal diet necessary to prevent histological scorbutic changes in the teeth of normal guinea-pigs [Höjer, 1926].

In the former experiment, the animals (male guinea-pigs, selected and grouped according to weight and previous growth) were fed on a scorbutic basal diet composed as follows: Sussex ground oats, 3600 g.; bran, 400 g.; dried egg-yolk, 200 g. 1 % of cod-liver oil was added daily and the diet mixed to a friable mass with water. At the onset of scurvy and sudden cessation of normal growth, daily doses of 10 cc. and 5 cc. of liver extract were administered.

For comparison, two other groups of animals were given 1 g. and 0·5 g. cabbage *per diem* respectively, and in addition 5 cc. autoclaved liver extract (120° for 1 hour, sufficient for destruction of vitamin C) to control any ill effect of the extract upon digestive processes *etc.* Control animals received (1) a fully curative dose of 5 g. cabbage in addition to the basal diet; (2) basal scorbutic diet only.

The composition of the basal diet and the arrangement of the groups were identical in the second experiment. The animals were killed on the 14th day of the experiment, histological examination of the teeth and ribs being then made.

The results are shown in the relevant columns of Table I. From this it is seen that 5 cc. of the extract compares closely in its effect with 1 g. of cabbage, while, by Höjer's method, it is seen that the minimum protective dose lies between 5 and 10 cc. of the extract.

2. Effect of freezing and storage in the frozen state upon the vitamin C content of sheep-liver.

Freezing produces an irreversible change in the structure of the liver, which is shown by the fact that the tissue, on thawing, is spongy to the touch and loses a considerable amount of free fluid. It is essential, therefore, to consider separately the effects on the vitamin C content of (1) freezing the liver, (2) storage in the frozen state.

A comparison was accordingly made of the antiscorbutic properties of

(*a*) fresh sheep-liver;

(*b*) control portions of sheep-liver frozen at an average temperature of −19° for 6 days (*i.e.* sufficiently long to ensure thermal equilibrium);

(*c*) sheep-liver frozen at an average temperature of −19° and stored at this temperature for 6 months.

Fresh sheep-livers were halved, placed in a chamber at −19° until the surface had hardened, sealed in greaseproof paper to prevent evaporation, and stored at the low temperature. The storage was started at periods of

6 months and 6 days before the commencement of the feeding experiments and continued so as to provide a constant daily supply of frozen half livers stored for the required periods. These were then thawed for 24 hours at an average temperature of 15°, and after disintegration through a sieve and addition of the collected "drip," were treated in the same way as the fresh liver for the preparation of aqueous extracts. Feeding experiments were carried out side by side with those described above.

Table I.

	Curative method				Preventive method		
			Post mortem findings			Histological condition	
Additions to basal scorbutic diet	No. of guinea-pig	Clinical	Ribs	Joints	No. of guinea-pig	Teeth	Ribs
5 cc. extract of fresh sheep-liver	1 *a*	Healthy, but inactive	Tr.	+	A_3	−	−
	1 *b*	Mild scurvy, died from pneumonia	−	Tr.	A_4	Tr.	−
	1 *c*	Very mild scurvy, died from pneumonia	Tr.	−			
5 cc. extract of sheep-liver stored at −19° for 6 days	2 *a*	Severe	+	+	D_1	+	−
	2 *b*	Severe	++	+	D_2	++	+
	2 *c*	Severe, died 16 days from onset of scurvy	+++	++			
5 cc. extract of sheep-liver stored at −19° for 6 months	3 *a*	Very severe, died 21 days from onset	+++	+++	E_1	+++	?
	3 *b*	Death from pneumonia 12 days from onset	.	.	E_2	++	−
	3 *c*	Death from scurvy 16 days from onset	++	++			
10 cc. extract of fresh sheep-liver	4 *a*	Completely cured; animal active	−	−	A_1	−	−
	4 *b*	Normal, death from pneumonia	Tr.	−	A_2	−	−
	4 *c*	Slight stiffness (death from gastro-intestinal infection	−	−			
10 cc. extract of sheep-liver stored at −19° for 6 days	5 *a*	Death from infection after 11 days	−	−	B_1	+	−
	5 *b*	Death from infection after 12 days	Tr.	Tr.	B_2	++	−
	5 *c*	Death from infection after 9 days	−	−			
10 cc. extract of sheep-liver stored at −19° for 6 months	6 *a*	Very severe	+++	+++	C_1	++	Tr.
	6 *b*	Very severe	++	+++	C_2	++	Tr.
	6 *c*	Very severe	++	++			
Positive control 5 g. cabbage	7 *a*	Normal	−	−	F	−	−
	7 *b*	Normal	−	−			
5 cc. autoclaved extract +1 g. cabbage	8 *a*	Mild scurvy	−	+	J	−	−
	8 *b*	Healthy but inactive	Tr.	+			
5 cc. autoclaved extract +0·5 g. cabbage	9 *a*	Severe scurvy, death 8 days from onset (pneumonia)	++	++	I	+	−
	9 *b*	Severe scurvy, death 12 days from onset	++	++			
Negative control	10 *a*	Very severe scurvy, death 10 days from onset	+++	+++	H	.	++
	10 *b*	Very severe scurvy, death 11 days from onset	+++	+++			

Degrees of scurvy: normal (−), trace (Tr.), mild (+), severe (++), very severe (+++).

The results of this comparison are shown in Table I and Figs. 1 and 2. It will be observed that there is a definite loss of antiscorbutic activity from sheep-liver which has been stored at −19° for 6 days and then thawed, for while the minimum protective dose (Höjer's method) lies between 5 and 10 cc. of the fresh liver extract, 10 cc. of the extract from frozen liver are not sufficient to prevent histological scorbutic changes from occurring in the teeth.

The loss after freezing for 6 days is much less than that observed in the extract of liver stored at −19° for 6 months, 5 cc. of the aqueous extract of the former being approximately equal in effect to 10 cc. of the latter.

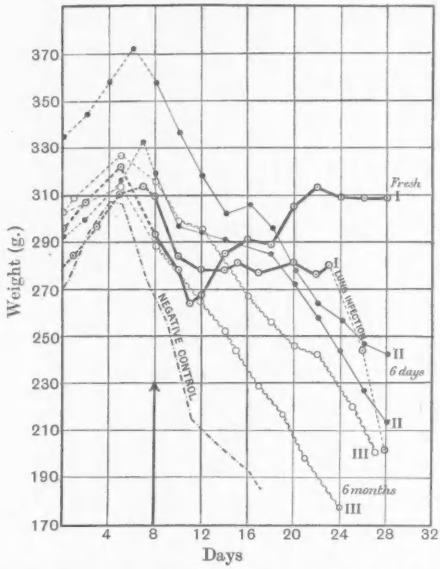

Fig. 1. Growth curves of guinea-pigs, showing the comparative curative power of 5 cc. aqueous extract of (I) fresh sheep-liver, (II) sheep-liver stored at average temperature of $-19°$ for 6 days, (III) sheep-liver stored at average temperature of $-19°$ for 6 months, when dosed to scorbutic animals receiving a vitamin C-free basal diet. (Arrow denotes onset of scurvy and commencement of administration of the sheep-liver extracts.)

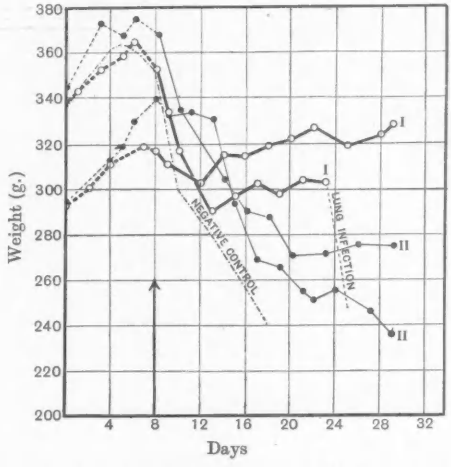

Fig. 2. Growth curves of guinea-pigs, showing the comparative curative effects of 10 cc. (*per diem*) of aqueous extracts of (I) fresh sheep-liver, (II) sheep-liver stored for 6 months at temperature of $-19°$. (Arrow denotes onset of scurvy and commencement of administration of sheep-liver extracts.)

3. Effects of slow and rapid freezing without storage.

The foregoing experiment was designed to distinguish between the separate effects of freezing and of storage in the frozen state, and the results indicated that a short period of freezing, followed by thawing, brings about a loss of vitamin C, while storage for a prolonged period in the frozen state causes further destruction. Further experiments were carried out to confirm the inactivating effect of freezing without prolonged storage and at the same time a comparison was also made of the effects of slow and rapid freezing.

Preparation of material. Fresh whole sheep-livers were procured daily, and from each of the two lobes a portion weighing 150–200 g. was cut to fit exactly into a rectangular tin of cross section 1×3 inches. The remainder was sealed in greaseproof paper to prevent evaporation, and stored in a chamber at 0°.

Slow freezing in air. One tin containing the sample of liver was packed in granulated cork to a depth of $1\frac{1}{2}$ inches in each direction, the exposed surface of the liver being covered by $1\frac{1}{2}$ inches of cotton-wool and the tin sealed with cork. Under these conditions a period of 21 hours was required for the sample to cool from $+10°$ to $-19°$ (measured by a thermocouple placed at the centre of the sample of liver). A further storage period of 24 hours at this temperature was allowed to ensure complete thermal equilibrium.

Rapid freezing in brine. The tin containing the second sample was suspended in well-stirred brine at $-19°$ (time of cooling from $+10°$ to $-19°$ 32 mins.) and after allowing a period of 80 minutes in the tank, was transferred to the $-19°$ chamber.

At the end of 48 hours the brine- and air-frozen samples were removed from the cold chamber and were thawed in air at $+10°$ for 22 hours[1]. Aqueous extracts of these, and of the unfrozen sample which had been stored for the same length of time at 0°, were then prepared and tested on guinea-pigs as in the previous experiment.

Feeding experiments. These consisted of an examination of the extracts by

(*a*) a measurement of their curative power when administered to scorbutic guinea-pigs in graded daily doses;

(*b*) a comparison of their power to prevent histological scorbutic changes in the teeth of normal guinea-pigs, the extract being administered in graded daily doses, for 14 days, in addition to the scorbutic basal diet.

The selection, grouping and treatment of the animals were exactly the same as in the previous experiment. The scorbutic basal diet contained, in addition to the aforementioned constituents, 1% mineral salt mixture.

The results of these investigations are shown in Table II and Fig. 3, and it will be seen that the growth changes, the *post mortem* observations, and the histological condition of teeth and ribs combine to prove that a definite loss of antiscorbutic activity occurs in sheep-liver which has undergone the pro-

[1] This is slow thawing. Experiment has not yet been made of the effects of rapid freezing followed by rapid thawing.

Table II.

Addition to basal scorbutic diet	Curative method: No. of guinea-pig		Curative method: *Post mortem* condition: Ribs	Curative method: *Post mortem* condition: Joints	Preventive method: No. of guinea-pig		Preventive method: *Post mortem* examination	Preventive method: Histological condition: Teeth	Preventive method: Histological condition: Ribs
5 cc. extract of sheep-liver stored at 0° (3 days)	IV	32	+	−	IV	10	Tr.	+	−
		33	−	+		11	−	+	−
		34	+	+		12	−	+	−
5 cc. extract of sheep-liver frozen slowly in air at −19°	V	35	+	+ +	V	13	Tr.	+ +	−
		36	+ + +	+ + +		14	Tr.	+ +	−
		37	−	+		15	Tr.	+ +	−
5 cc. extract of sheep-liver frozen rapidly in brine at −19°	VI	38	+ +	+ + +	VI	16	+	+ +	−
		39	+ +	+ + +		17	−	+	−
		40	+ +	−		18	−	+	−
10 cc. extract of sheep-liver stored at 0° (3 days)	I	23	−	−	I	1	−	−	−
		24	−	−		2	−	−	−
		25	−	−		3	−	−	−
10 cc. extract of sheep-liver frozen slowly in air at −19°	II	26	+	−	II	4	−	−	−
		27	−	−		5	−	−	−
		28	+	−		6	−	−	−
10 cc. extract of sheep-liver frozen rapidly in brine at −19°	III	29	Tr.	−	III	7	−	−	−
		30	Tr.	−		8	−	−	−
		31	−	−		9	−	−	−
Negative control	VII	43	+ + +	+ +	VII	19	+ + +	+	+ +
		44	+ + +	+ +		20	+ +	+	+
Positive control, 5 g. cabbage	VIII	41	−	−	VIII	21	−	−	−
		42	−	−		22	−	−	−

Degrees of scurvy: normal (−), trace (Tr.), mild (+), severe (+ +), very severe (+ + +).

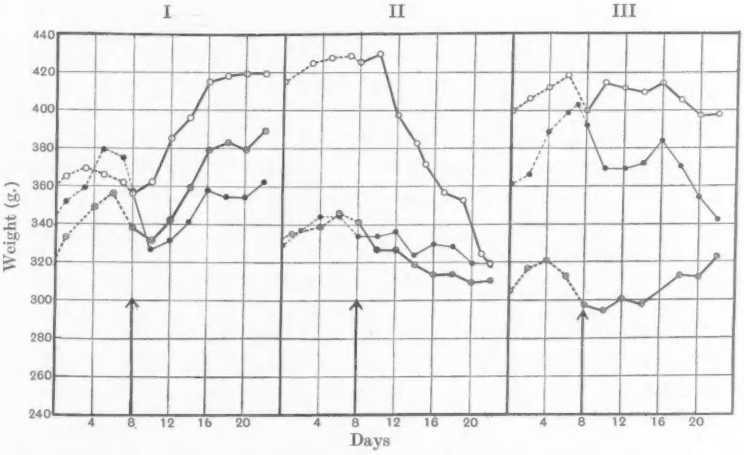

Fig. 3. Growth curves of guinea-pigs, showing the antiscorbutic activities of 5 cc. aqueous extracts of sheep-liver (I) kept at zero without freezing for 3 days, (II) frozen rapidly to −19°, stored for 48 hours at temperature of −19°, (III) frozen slowly to −19°, stored for 48 hours at temperature of −19°. (Arrow denotes onset of scurvy and commencement of administration of sheep-liver extracts.)

cesses of freezing and subsequent thawing. Further, in regard to the method of freezing, this loss is as great after rapid freezing in brine at $-19°$ as after slow freezing in air at the same temperature.

Discussion.

These experiments demonstrate the high antiscorbutic activity of sheep-liver, of which the minimum protective dose lies between 5 and 10 cc. of the extract (*i.e.* between 2·88 and 5·77 g. of the liver, on the assumption made above). This is in agreement with the results of work on the vitamin C content of the livers of other animals.

It has been rendered probable that the rat, the chick, the calf (see references on p. 704) and also that puppies [Harris and Innes, 1931] can synthesise vitamin C and that the livers of these animals have a comparatively high antiscorbutic potency. It is possible, though yet to be proved, that sheep may also have this synthetic power.

The marked loss of antiscorbutic activity caused by the process of freezing and thawing is probably associated with the structural breakdown of the liver tissue brought about through the formation of ice crystals which may act directly by causing mechanical damage or indirectly by concentrating the aqueous phase in frozen liver; it is the latter which is responsible for the changes in living muscle when it is frozen. This disintegration may well lead to the liberation into the free fluid of enzymes or other substances capable of inactivating the vitamin.

The mere lowering of temperature must retard any chemical changes in the vitamin and thus assist in its preservation.

As rapid freezing leads to the formation of smaller ice crystals in the tissue than does slow freezing it should cause a less degree of structural breakdown. No appreciable difference could, however, be observed between the vitamin C contents of slowly and of rapidly frozen liver.

During storage for 6 months in the frozen state a further loss takes place. This may be due to further structural breakdown in the tissue and consequent liberation of substances capable of inactivating the vitamin on thawing. Attention may here be drawn to the work of Bracewell *et al.* [1931] on the effect of cold storage on the vitamin C content of apples. It was found by these workers that prolonged storage at low temperatures had no effect on the antiscorbutic potency of apples. However, the stability of vitamin C has been shown to vary in different media; among other factors an increase in the hydrogen ion concentration of the medium is associated with increased stability of the active constituent [Holst and Frölich, 1912]. It is possible that vitamin C is more stable in apples than in sheep-liver during cold storage on account of the greater acidity of the former material.

SUMMARY.

1. Sheep-liver contains an appreciable amount of vitamin C.

2. The mere act of bringing the liver into the frozen condition followed by thawing brings about a considerable decrease in its vitamin C activity.

3. Still further loss occurs from liver which is stored at $-19°$ for 6 months.

4. It is suggested that the structural breakdown caused by freezing liberates, on thawing, substances from the liver tissue which have a destructive action on the vitamin.

5. No appreciable difference was observed between the effect of rapid freezing and slow freezing on the vitamin C content of sheep-liver.

The experimental material was provided and the material prepared at the Low Temperature Research Station where Dr T. Moran gave me every assistance. The feeding experiments were carried out at the Nutritional Laboratory, Cambridge, under the supervision of Dr L. J. Harris. I am also indebted to Dr J. R. M. Innes for the histology.

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LXXXVIII. HYDROGENLYASES. BACTERIAL ENZYMES LIBERATING MOLECULAR HYDROGEN.

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(*Received April 28th, 1932.*)

The earliest work dealing with the bacterial decomposition of formic acid was that of Hoppe-Seyler [1876], who dealt with its decomposition into carbon dioxide and hydrogen by mixed cultures obtained from mud, and showed that calcium formate was decomposed according to the equation

$$(H.COO)_2.Ca + H_2O \rightarrow CaCO_3 + CO_2 + 2H_2.$$

Later Pakes and Jollyman [1901, 1] showed that without exception those organisms which, in the presence of peptone water, fermented sugars with the production of hydrogen, also decomposed formic acid into carbon dioxide and hydrogen, and they drew the conclusion that the hydrogen produced in the fermentation of sugars arose from the decomposition of intermediately formed formic acid. The evidence of Harden [1901], Grey [1914] and many others confirmed this view, and it has been considered established that the hydrogen from sugar fermentations arises solely from decomposition of formic acid.

Thus the earlier work dealing with the bacterial attack on formic acid concerned itself entirely with the production of molecular hydrogen. Along with the study of bacterial dehydrogenases a second mode of attack on formic acid came into view [Quastel and Whetham, 1925]. Here it was shown by the use of washed suspensions of bacteria and the methylene blue technique that there existed an enzyme catalysing the reaction

$$H.COOH + X = CO_2 + XH_2$$

where X represents, in the experiments, methylene blue, and, in the normal metabolism of the cell, probably an intracellular hydrogen carrier. The behaviour of formic acid in this reaction is the same as that of a large number of other substances which donate hydrogen to acceptors but do not give off molecular hydrogen.

Stickland [1929] attempted to find the relation between these two modes

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of decomposition of formic acid by *Bact. coli*. He found that washed suspensions rapidly dehydrogenated formic acid in the presence of methylene blue, but did not carry out the reaction resulting in the liberation of hydrogen gas until they had been in contact with the formate solution for some 24 hours. Viable cell counts showed that the appearance of hydrogen did not begin until the surviving cells had begun to grow on the autolysed remains of the dead ones. This was shown more conclusively in an experiment in which the initial viable count was reduced to 0·0001 % of its former value by the action of ultra-violet light; the evolution of hydrogen coincided with the growth that took place after the dead cells had autolysed. A provisional theory was put forward suggesting that the production of molecular hydrogen was a function associated with the actual growth of the cells, but the true explanation will be made clear in the course of this paper.

This re-investigation of the subject is due to a stimulus received by the present authors from the recent work of Karström [1930]. The latter, confirming the early work of Dienert [1900] on yeast, has shown conclusively that, among the bacterial enzymes (or enzyme systems) attacking the sugars, some form an invariable part of the make-up of the cell (these he terms *constitutive* enzymes), while others are definitely absent unless the cells have actually been grown in the presence of the substrate (*adaptive* enzymes). For instance, *Bact. aerogenes*, grown in whey and washed and suspended in saline, failed to ferment xylose; if a source of nitrogen, permitting the growth of the washed cells, were added, fermentation set in after a varying latent period. If on the other hand the organism were grown on a medium containing xylose, fermentation of this sugar by the washed suspension set in immediately. Fermentation of glucose by washed suspensions was independent of the medium on which the cells had been grown, so that the glucose enzyme was constitutive, the xylose one adaptive.

Karström's paper recalled to us the fact that the cell suspensions used in Stickland's work on formate decomposition had been grown on tryptic broth in the absence of formate; it seemed possible that the introduction of formate into the medium might so modify the cells that a washed suspension would be able to decompose formate into carbon dioxide and hydrogen, and allow of a study of the enzyme concerned apart from growth.

The production of the enzyme formic hydrogenlyase.

The organism used was *Bact. coli* (Escherich) grown for not more than 20 hours in Roux bottles containing tryptic caseinogen broth at p_H 7 with or without 0·5 % sodium formate. The cells were centrifuged out and washed twice with saline or Ringer's solution and finally uniformly suspended in the same solution. The evolution of hydrogen was measured in the Barcroft differential manometer in the usual way, the cups being twice evacuated and filled with nitrogen purified by passage over heated copper, and the apparatus being shaken in a bath at 40°.

Fig. 1 gives the result of a typical experiment, and shows clearly that the cell suspension grown in broth *plus* formate subsequently decomposes formate with evolution of hydrogen, while that grown on plain broth does not. The presence of hydrogen in the cup at the end of the experiment was verified on several occasions by analysis of a sample of the gas in the Haldane apparatus.

Before definitely attributing this difference to the presence of formate in the broth, two other possibilities which might account for its occurrence must be eliminated, *viz.* the facts that (1) the decomposition of the formate renders the medium practically anaerobic, (2) the base liberated from the sodium formate makes the medium progressively more alkaline.

(1) If the hydrogenlyase were labile to oxygen, aerobic conditions during growth would prevent its appearance, and the formate might act merely by

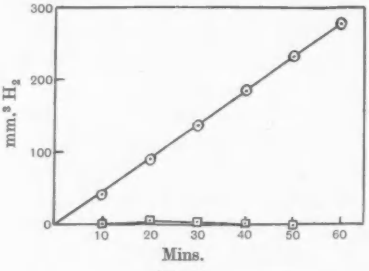

Fig. 1.

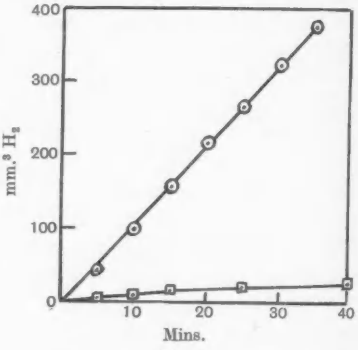

Fig. 2.

Fig. 1. Action on *M*/30 formate at p_H 7·0 of suspensions of *Bact. coli* grown aerobically. ⊙——⊙ in presence of formate. ⊡——⊡ in absence of formate.

Fig. 2. Action on *M*/30 formate at p_H 7·0 of suspensions of *Bact. coli* grown anaerobically. ⊙——⊙ in presence of formate. ⊡——⊡ in absence of formate.

keeping the medium partly anaerobic. Actually aeration of a suspension possessing the enzyme has little harmful effect:

	Velocity (mm.³ H_2/hour)
Original suspension	580
Suspension after 45 mins. vigorous aeration at 20°	495

A further test was carried out by growing cultures on plain broth and formate broth under strictly anaerobic conditions, and testing the evolution of hydrogen by the washed suspensions as before. The results (Fig. 2) show that the organisms grown anaerobically on plain broth give a slow evolution of hydrogen, very small in comparison with that produced by the control suspension grown anaerobically on formate broth. This disposes of the possibility that anaerobiosis can account for the previous results. The slow decomposition by the suspension from plain anaerobic broth might be due to traces of formic acid known to be produced from the amino-acids of the broth.

(2) The final p_H of a broth medium containing 0·5 % sodium formate on which *Bact. coli* has been growing for 20 hours is about 8·0 or higher. That this change of p_H accounts for the production of the hydrogenlyase is contradicted by the fact, to be shown later, that cells grown in the presence of glucose also possess the enzyme, although the change in reaction is here in the opposite direction, the medium reaching a final p_H of 5·5–6·0.

We therefore conclude that the enzyme in question is an adaptive one in the sense used by Karström, and occurs as the result of growing the cells in the presence of formate. We propose the name *hydrogenlyase* for enzymes which liberate molecular hydrogen, to distinguish them from the dehydrogenase type; the evidence for the existence of different hydrogenlyases will be presented later.

The production of formic hydrogenlyase by growth on substances other than formate.

The introduction of glucose or glycerol (0·5 %) into the broth also results in the production of a suspension containing formic hydrogenlyase. As is well known, however, both these substrates are decomposed by *Bact. coli* with production of formic acid [Harden, 1901; Braak, 1928], so this phenomenon is sufficiently explained.

Some properties of formic hydrogenlyase.

(1) Fig. 3 gives the rate of action of the enzyme acting on *M*/30 formate, plotted against p_H, and needs no further comment. All other experiments were done at the optimum, p_H 7·0.

(2) Fig. 4 shows the affinity curves of formic dehydrogenase and formic hydrogenlyase (velocity plotted against logarithm of substrate concentration). The much lower affinity of the latter enzyme will be noted.

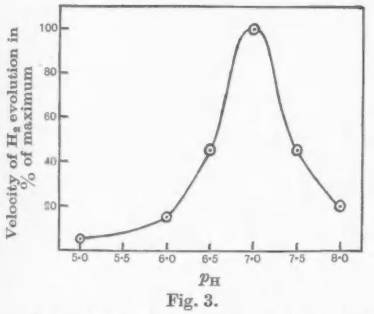

Fig. 3.

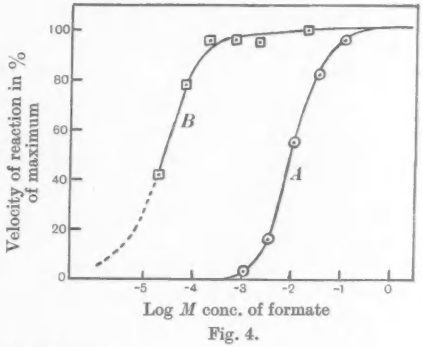

Fig. 4.

Fig. 3. Effect of p_H on action of formic hydrogenlyase.

Fig. 4. Affinities of *A*, ⊙———⊙ formic hydrogenlyase; *B*, ⊡———⊡ formic dehydrogenase.

(3) The enzyme is very labile to poisons of all kinds. Toluene, 1 % sodium fluoride, 2 % urethane and carbon monoxide at one atmosphere pressure all cause 100 % inhibition. Hydrogen from a Kipp's apparatus gave 100 % inhibition even at 0·2 atmosphere pressure, but this was found to be due to a trace of some poison which was not removed by passing the gas through silver nitrate solution, since hydrogen from a cylinder gave a much smaller inhibition (about 40 % at 1 atmosphere); this latter, and the inhibition by carbon monoxide mentioned above, may also have been due to some impurity not removed by passing the gas through silver nitrate solution or over heated copper, but the point has not been further investigated.

The enzyme is also very sensitive to the action of cyanide, 50 % inhibition being produced by a concentration of 10^{-5} *M*, as is shown in Fig. 5.

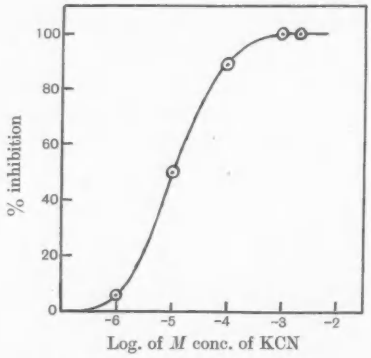

Fig. 5. Effect of cyanide on formic hydrogenlyase.

(4) Pakes and Jollyman [1901, 2] noted that, in the presence of nitrate, bacteria which normally decompose formate into hydrogen and carbon dioxide no longer do so, but instead oxidise the formate with reduction of nitrate to nitrite. We shall show later that there is no connection between formic dehydrogenase and formic hydrogenlyase, so it is difficult to see why the presence of a hydrogen acceptor should completely suppress the formation of hydrogen; we should expect the two enzymes to work side by side.

When oxygen is used as the hydrogen acceptor, *i.e.* when a suspension of bacteria containing formic hydrogenlyase is placed with formate in a Barcroft manometer filled with air there are three reactions taking place simultaneously, *viz.*

$$H.COOH \rightarrow H_2 + CO_2,$$

$$HCOOH + \tfrac{1}{2}O_2 \rightarrow CO_2 + H_2O,$$

and

$$H_2 + \tfrac{1}{2}O_2 \rightarrow H_2O.$$

The manometer readings express the balance of these three reactions, and it is impossible to deduce what is happening. In actual experiments the change

of pressure was practically nil, showing that the hydrogen given off and the oxygen taken up roughly balanced. Without enormous complications of experimental procedure nothing more can be learnt on this point.

With nitrate as the hydrogen acceptor the measurements might have been easier, as initially only one gas reaction is taking place, *viz.*

$$H.COOH \rightarrow H_2 + CO_2.$$

Eventually however the reaction

$$KNO_3 + H_2 \rightarrow KNO_2 + H_2O$$

will produce an apparent inhibition, and more important still the reaction

$$H.COOH + KNO_3 \rightarrow CO_2 + KNO_2 + H_2O$$

is rapidly making nitrite which gradually poisons the formic hydrogenlyase. Hence the measurement of velocity of hydrogen production in the presence of nitrate is not very accurate, but an estimate may be made before the nitrite poisoning becomes very appreciable.

The results of determinations of velocities of hydrogen production at different concentrations of formate and nitrate are given in Table I. It will be seen that the amount of inhibition varies very little with either the formate concentration or the nitrate concentration; the complete inhibition reported by Pakes and Jollyman is not observed.

Table I.

Formate concentration	Nitrate concentration: 0	*M*/60	*M*/180	*M*/600	*M*/6000	Inhibition %
	Velocity (mm.³ H_2/hour)					
M/6	456	156	144	—	—	67
M/12	480	216	—	228	—	54
M/15	354	90	—	108	84	73
M/30	1020	432	—	384	360	62
M/60	756	264	—	304	—	62

It has been stated already that this effect of nitrate cannot be due to its action as a hydrogen acceptor, as formic dehydrogenase and formic hydrogenlyase are completely separate enzymes (direct experimental confirmation of this might have been obtained by inhibiting nitrate activation by potassium cyanide but for the fact that cyanide inhibits formic hydrogenlyase even more strongly than it does nitrate activation). It seems therefore that the inhibiting effect of nitrate on the reaction

$$H.COOH \rightarrow H_2 + CO_2$$

is most probably due to a direct combination with the formic hydrogenlyase, the formate thus being prevented from reaching the enzyme. This reminded us of the work of Mann and Woolf [1930] on the action of salts on fumarase, in which various salts acted by changing the p_H-activity curve of the enzyme. Similar experiments to those of Mann and Woolf were done on formic hydrogenlyase, the salts used being nitrate, sulphate, fumarate, citrate and oxalate;

nitrate and sulphate produced the effects shown in Fig. 6, the others having no effect at all. It is significant that sulphate, which is not activated as a hydrogen acceptor, inhibits, while fumarate, which is activated as a hydrogen acceptor, does not. We consider that the action of nitrate and sulphate is similar to the salt effects described by Mann and Woolf with fumarase, and that the results of Pakes and Jollyman were probably due to destruction of the formic hydrogenlyase by the nitrite formed during their experiments.

(5) The theoretical amount of hydrogen is obtained when *Bact. coli* acts on known quantities of formate. For these experiments the substrate was placed in a small tube hooked on the small cup of the Barcroft apparatus, and tipped into the suspension of bacteria after the apparatus had reached equilibrium in the bath.

Amount of formate cc. *M*/10	H_2 (measured) mm.³	H_2 (theoretical) mm.³	% yield
0·5	1110	1120	99
0·2	472	448	106
0·2	448	448	100
0·1	218	224	97

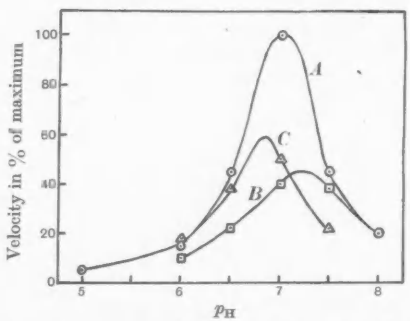

Fig. 6. Effect of salts on p_H-velocity curve of formic hydrogenlyase.
A. ⊙——⊙ normal. *B*. ⊡——⊡ $+KNO_3$ *M*/30. *C*. ◬——◬ $+Na_2SO_4$ *M*/30.

The production of hydrogen from glucose.

As *Bact. coli* is able to ferment glucose with formic acid as one of the decomposition products, it is to be expected that a suspension will liberate hydrogen from glucose also, in the same circumstances as it does from formate. This expectation is realised (Fig. 7).

It has generally been held that organisms fermenting sugars with production of hydrogen do so by virtue of the fact that formic acid is an intermediate product. We are now in a position to investigate this hypothesis more closely. The questions to be answered are (1) does the hydrogen arising by the action of a washed suspension of *Bact. coli* on glucose come from formic acid produced by preliminary fermentation of the sugar, or does it come from glucose direct or perhaps from some other intermediate product? and (2) in the latter case are we dealing with the same enzyme acting on two different substrates

(formic acid and glucose or some other intermediate compound) or with two separate enzymes?

With regard to the first question the evidence is conclusively against the view that hydrogen comes from glucose by decomposition of preliminarily formed formic acid. In the first place the production of hydrogen from glucose proceeds linearly and is not subject to any lag, as would be expected if a preliminary decomposition to formic acid were taking place, especially considering the low affinity of formic hydrogenlyase. Secondly, the affinity of the enzyme liberating hydrogen from glucose is very much higher than that of formic hydrogenlyase; the affinities of the two enzymes are compared in Fig. 8, that of the glucose enzyme being too large to be measured. At low

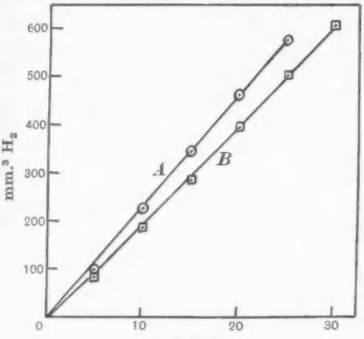

Fig. 7.

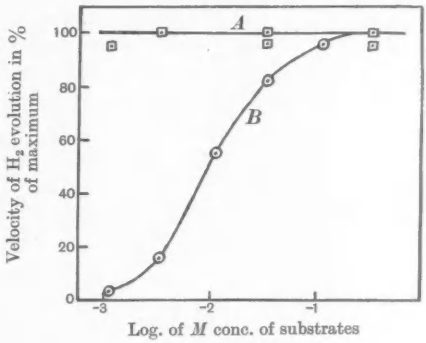

Fig. 8.

Fig. 7. Action of suspension of *Bact. coli* grown in presence of formate on *A*, ⊙———⊙ *M*/30 formate at p_H 7·0; *B*, ⊡———⊡ *M*/30 glucose at p_H 6·2.

Fig. 8. Comparison of affinity of glucose hydrogenlyase at p_H 6·2 (*A*, ⊡———⊡) with that of formic hydrogenlyase at p_H 7·0 (*B*, ⊙———⊙).

substrate concentrations the rate of evolution of hydrogen from glucose is actually greater than that from the same molar concentration of formate, *e.g.*

	Velocity (mm.³/hour)
From *M*/300 formate at p_H 7·0	252
,, glucose ,,	360

These facts taken in conjunction make it quite certain that the hydrogen liberated from glucose does not arise by the intermediate formation of formic acid.

The second question now arises, *viz.* whether the same enzyme acts on glucose and on formate, or whether there is a separate specific hydrogenlyase for each. This question may be investigated in two ways; first, by so treating the suspension that it contains one enzyme but not the other; second, by determining whether, in a saturating concentration of both substrates, the rate of evolution of hydrogen is equal to the greater of the rates on the two substrates separately, or to their sum.

The former of these experiments gave results indicating two specific enzymes for the two substrates. A suspension grown on formate broth liberates hydrogen from formate at p_H 7·0 at about the same rate as it does from glucose at p_H 6·2 (Fig. 7). A suspension grown aerobically on plain broth, as has already been shown, does not decompose formate, but this suspension is able to bring about a slow liberation of hydrogen from glucose (Fig. 9). The ratio

$$\frac{\text{rate of liberation of } H_2 \text{ from glucose}}{\text{rate of liberation of } H_2 \text{ from formate}}$$

is infinitely greater in the latter suspension than it is in the former, and this fact is not consistent with the theory that the same enzyme catalyses both reactions.

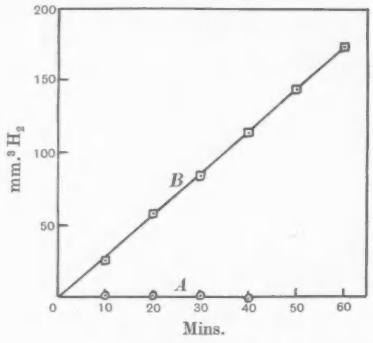

Fig. 9.

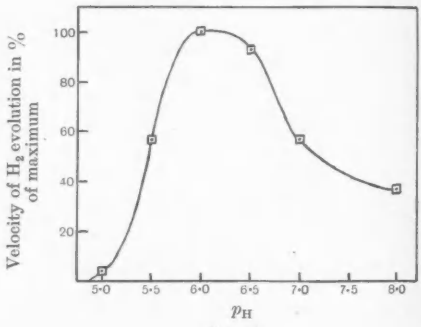

Fig. 10.

Fig. 9. Action of suspension of *Bact. coli* grown aerobically in absence of formate on *A*, ⊙——⊙ *M*/30 formate, p_H 7·0; *B*, ⊡——⊡ *M*/30 glucose, p_H 6·2.

Fig. 10. Effect of p_H on glucose hydrogenlyase.

The second line of investigation has given results pointing to the same conclusion. It is difficult to choose the right conditions for measuring the effect of the two substrates together, as the two reactions have different p_H optima, that for formate being at p_H 7·0 and that for glucose at p_H 6·2 (the relation between p_H and velocity of liberation of hydrogen from glucose is given in Fig. 10; that for formate is given in Fig. 3). Experiments done at p_H 7·0 and p_H 6·2 both show that the velocity of hydrogen evolution from a mixture of the two substrates is equal to the sum of the velocities from the two separately. The present state of our knowledge on this point is therefore that

	Velocity (mm.³ H_2/hour)	
(1) At p_H 7·0. Formate *M*/30	300	363
Glucose *M*/30	63	
Formate *M*/30 + glucose *M*/30		348
(2) At p_H 6·2. Formate *M*/30	234	411
Glucose *M*/30	177	
Formate *M*/30 + glucose *M*/30		413

the hydrogen from glucose does not come through formic acid and that it is liberated by an enzyme which is not formic hydrogenlyase; whether it is liberated direct from the glucose molecule or from some other intermediate compound we do not know, though the absolute linearity of the reaction indicates that the former may be true.

One further piece of evidence has been obtained about the origin of this hydrogen. If *Bact. coli* is allowed to act on glucose in two Barcroft manometers, one with potash in the small cup and the other without, the rate of gas production is greater in the latter, and this must indicate simultaneous evolution of carbon dioxide. If acid is spilt into the solutions after a given period of reaction, a further quantity of carbon dioxide is liberated, the total excess over the experiment where the carbon dioxide is absorbed representing roughly one molecule of carbon dioxide per molecule of hydrogen. This does not at present throw any light on the chemistry of the reaction.

In all recent schemes setting out the course of hexose fermentation by bacteria of the *coli-typhosus* group it has been assumed that the hydrogen appearing among the fermentation products arises from intermediately formed formic acid. It is now clear that this is not necessarily the case since hydrogen may be liberated from glucose by bacterial preparations which are unable to produce hydrogen from formate. The origin of this hydrogen is still obscure. In our experiments it was always attended by a simultaneous evolution of carbon dioxide in roughly equimolecular proportions; it seems therefore to be of the nature of a decarboxylating process but whether acting on a six carbon compound or on some unstable product of smaller carbon chain is not clear.

The production of hydrogen from other substances.

Glucose, fructose and mannose all yield hydrogen at about the same rate; lactose, galactose, arabinose, glycerol and mannitol all react much more slowly; sucrose, lactate and succinate do not react at all.

Glucose, fructose and mannose appear to act at the same enzyme, for mixtures of two substrates do not produce hydrogen faster than either substrate alone:

	Velocity (mm.³/hour)	
	(1)	(2)
Glucose *M*/60	168	135
Mannose *M*/60	126	—
Glucose *M*/60 + mannose *M*/60	162	—
Fructose *M*/60	—	140
Glucose *M*/60 + fructose *M*/60	—	144

It is possible that the other substances, and even other sugars not so closely related structurally as these three, may require other specific enzymes, especially as different bacterial species have very different powers of fermenting various sugars and alcohols, but we have not yet tested this point experimentally.

Comparison of rates of action of hydrogenlyases with those of dehydrogenases.

The dry weight of bacteria present in many of the suspensions has been estimated by determination of total nitrogen. By this means the dry weight of bacteria in any suspension may be calculated from a small sample, and the waste of material involved in the direct estimation is avoided. The nitrogen content of the dried bacteria is the same whether the suspensions are grown with or without formate:

	N content %	Average %
Suspension grown on plain broth	12·0, 13·5	12·7
„ „ formate broth	12·1, 11·7	11·9
		12·3

Our values are all higher than that quoted for *Bact. coli* (10·3 %) by Nicolle and Alilaire [1909]. To express the results the symbol Q_{H_2} has been used with a meaning analogous to that of the widely used Q_{O_2}, *viz.* mm.^{3}/hour/mg. dry weight of cells. The values of Q_{H_2} obtained varied over a rather wide range, *viz.* for formate 340–960, with an average of 615 (6 determinations) and for glucose 170–400, with an average of 280 (5 determinations), the reaction being carried out at a substrate concentration of $M/30$, at 40° and at the optimum p_H. This variation is perhaps to be expected with adaptive enzymes, and could not be correlated with any controllable factor operating during the growth of the organisms.

For comparison with these values of Q_{H_2} for formate some estimations of Q_{O_2} for the same substrate were made on suspensions grown in the absence of formate (these values could not be obtained for suspensions containing formic hydrogenlyase). The average for two experiments gave Q_{O_2} (formate) = 225, and, allowing for the fact that formic acid gives off two atoms of hydrogen while its oxidation requires only one atom of oxygen, this means that the decomposition of formate into carbon dioxide and hydrogen takes place rather faster than its oxidation, when the appropriate suspensions of *Bact. coli* are used. Our value of Q_{O_2} (formate) = 225 is considerably higher than that of 40 found by Cook and Haldane [1931].

For suspensions grown aerobically on plain broth the Q_{H_2} for formate was always zero, while the slow evolution of hydrogen from glucose by these suspensions gave values of 31 and 42 (about 10–15 % of the normal average value of 280 obtained when the organism is grown in the presence of formate).

The relation between hydrogenase, formic dehydrogenase and formic hydrogenlyase.

Three bacterial enzymes are now known which act on formic acid or hydrogen:

(1) hydrogenase, which activates molecular hydrogen

$$H_2 + X \rightleftharpoons XH_2;$$

(2) formic dehydrogenase, which catalyses the reaction

$$H.COOH + X \rightleftharpoons XH_2 + CO_2;$$

(3) formic hydrogenlyase, which catalyses the reaction

$$H.COOH \rightleftharpoons H_2 + CO_2.$$

where X represents an intracellular hydrogen carrier, or experimentally a dye such as methylene blue. In a former communication [Stephenson and Stickland, 1931] we suggested the possibility that reaction (3) might be a combination of reactions (1) and (2), pointing out that such a view would be contradicted by the existence of an organism possessing the enzyme (3) but lacking (1) or (2), or even possessing both (1) and (2) but lacking (3). We have now further investigated the distribution of these enzymes, and the relevant results are summarised in Table II.

Table II.

Species	Medium grown on	Hydrogenase	Formic dehydrogenase	Formic hydrogenlyase
Bact. coli	Broth	+	+	0
Bact. coli	,, +formate	+	+	+
Bact. lactis aerogenes (4 strains)	,,	0	+	0
Bact. lactis aerogenes (4 strains)	,, +formate	0	+	+
Bact. dispar	,, +formate	+	+	0

Hydrogenase was tested for by putting the suspension in a Thunberg tube with 1 cc. 1/5000 methylene blue at p_H 6·5, filling the tube with hydrogen and noting the reduction time; formic dehydrogenase by adding 1 cc. $M/10$ sodium formate to a similar tube and simply evacuating and taking the reduction time; and formic hydrogenlyase by the method already described in this paper. The negative result for hydrogenase with *Bact. lactis aerogenes* might have been due to inability of the dye to reach the enzyme, though this was improbable since the formic dehydrogenase was always very vigorous in the same suspensions. This result was however of the greatest importance, so it was confirmed by testing the reaction

$$H_2 + KNO_3 = H_2O + KNO_2.$$

There can be no question whether hydrogen was able to permeate the cell, for hydrogen was given off copiously in the formic hydrogenlyase test; moreover, nitrate was rapidly reduced by other donators, so that if hydrogenase were present there should have been rapid uptake of hydrogen in the presence of nitrate. Experimentally, with *Bact. lactis aerogenes* there was none; so we feel justified in saying that hydrogenase is absent.

From the data given above it can be seen that all strains of *Bact. lactis aerogenes* when grown in presence of formate possess formic hydrogenlyase but no hydrogenase; conversely, *Bact. coli* grown on plain broth and *Bact. dispar* even when grown with formate present have hydrogenase and formic

dehydrogenase but no formic hydrogenlyase. Thus their distribution, in conjunction with the other evidence, makes it certain that these three enzymes are distinct entities.

Summary.

1. The enzyme liberating molecular hydrogen from formic acid is distinct from formic dehydrogenase and from hydrogenase. The name formic hydrogenlyase is proposed for this enzyme.

2. In the case of *Bact. coli* formic hydrogenlyase is an adaptive enzyme, *i.e.* it is formed only when the organism is grown in the presence of formate.

3. Some properties of formic hydrogenlyase are recorded.

4. Formic hydrogenlyase has been shown to be distinct from the hydrogenlyases liberating hydrogen from the sugars.

5. In the conditions of the experiments here reported the evolution of hydrogen from glucose does not occur through formic acid as an intermediate product.

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LXXXIX. OXIDATIONS BY THE BRAIN.

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(*Received April 30th, 1932.*)

A FULL consideration of the work which has been carried out in relation to the causation and treatment of abnormal mental states shows that it is extremely desirable that researches be undertaken to discover how organic conditions, in psychotic states, differ from those existing in the normal. Much work has been carried out from a histological standpoint—particularly by Nissl, Alzheimer, Southard, Dunlop, Mott, Spielmeyer to quote only a few of the authors of many painstaking investigations. The results definitely suggest, in the very widespread disease of dementia praecox, organic changes occurring particularly in the nervous system. Mott [1920] states that the essential morbid change is one of nuclear decay most marked in the cortex and suggests that the histological findings are associated with deficient oxidation. Koch and Mann [1909] had already found, from analyses of the condition of sulphur in the brain, evidence of deficient oxidations. These results refer to dementia praecox. There is no question but that there occur marked histological changes in frankly organic mental diseases such as general paresis, which is of venereal origin, and treatment is on definitely organic lines, as in the clearing up or partial clearing up of general paresis by the action of malaria.

It is evident, however, that present histological methods of approach are most inadequate to show differences involving even considerable biochemical abnormalities. The fault lies in the technique. Histological methods involve the use of fixing and almost inevitably that of staining. Hardening a tissue in alcohol or treatment with the usual fixing agents produces changes in the tissue constituents which are greater than would ever be supposed to occur in the tissue when in the living condition. Dyestuffs are, in many instances, toxic to oxidising and even hydrolysing enzymes [Quastel and Wheatley, 1931, 2; Quastel, 1931]. It is not to be supposed that a fixed and stained section gives a true picture of the condition of a tissue in the living state. Morphological changes will be shown as well as the occurrence of foreign structures—but in an abnormal mental condition where possibly only relatively small differences from the normal are to be expected in the dynamics of the cell processes, the changes inherent in histological technique would obscure what changes may have been already present.

The alternative to the histological method of investigation is to examine the tissue along biochemical lines where every effort is made to preserve the tissue in the state in which it was present in the body and to determine by dynamic methods what differences exist between the tissues of abnormal mental states and those of the normal.

Work has been proceeding in this laboratory for the past two years on these lines—attention being focused on suitable methods to be used for comparative purposes. The lack of information on the biochemistry of the brain has made it imperative to study the tissues of normal animals before it has become profitable to make any study of the tissues of abnormal mental cases.

Histological work, as already mentioned, having pointed to the possibility of deficient oxidations in the cortex of certain psychotic cases, an endeavour has been made to study the oxidative processes of normal brain with a view to obtaining quantitative data which may be used subsequently for comparative purposes. The attempt has been made also to study these processes when brain is submitted to various environmental conditions, including those brought about by the presence of narcotics. This paper will be concerned with a description of normal oxidative processes in brain whilst a subsequent paper will indicate the influence of narcotics in general on these processes.

Technique.

Velocities of oxidation by brain tissue were determined in a Barcroft differential manometer, all experiments being carried out at 37°. The whole brain was used in the cases of small animals such as mice, rats, guinea-pigs, whilst the grey matter of cerebral cortex was used with larger animals. In all cases the *pia* was removed as carefully and completely as possible from the brain of the freshly killed animal. One of the most serious difficulties in working with human material is to obtain material less than 6 hours old. Animals were in most cases killed by bleeding, the brain being used as quickly as possible. Storage of the brain, when circumstances made this necessary, was always at 0°. The brain, after removal of the *pia*, was sliced with a scalpel and made into as homogeneous a mixture as possible. The homogeneity was shown by the identity of velocities of O_2 uptake by equal quantities of the mixed tissue. Many control experiments showed that this method of preparing tissue for examination gave the most consistent and reproducible results, the O_2 uptake of the tissue per unit quantity being the same whatever amount was taken. Experience showed that 0·5 g. was the most useful quantity to work with and in all experiments this amount has been used. Ashford and Holmes [1931] have shown that brain used in this way gives results in every way comparable with sliced tissue and our experience has been such as to confirm this and to indicate that chopped brain suffers no material damage, so far as its oxidative mechanisms are concerned, by the process of chopping.

The tissue was immersed in a phosphate buffer solution p_H 7·4 containing 0·6 % saline. Values of the rate of O_2 uptake of a normal guinea-pig brain showed no significant differences when examined in this way from those obtained with a tissue suspended in Ringer solution[1] [see also Ashford and Holmes, 1931]. Thus 0·5 g. of guinea-pig brain took up 871 mm.^{3} O_2 in a phosphate saline medium in 2 hours and 861 mm.^{3} O_2 in the same time in the Ringer medium. Phosphate buffer solution has been used in all except special cases to remove all possibility of appreciable p_H changes during the course of oxidation and to ensure the presence of an adequate supply of phosphate ions.

The procedure has usually been to place in the right-hand vessel of the manometer 1 cc. $M/5$ phosphate buffer p_H 7·4, 2 cc. saline (0·9 %) and 0·5 g. tissue, and the same with the exception of the tissue in the left-hand vessel. The apparatus is shaken at the temperature of the bath for 10 mins. to ensure temperature equilibrium, the taps are closed and the manometer levels are read every 15 mins. When substrates have been added to the tissue, the same volumes in the manometer vessels are preserved and care taken that no serious change in ionic concentration is effected by the addition of the substrate.

Autoxidation of brain.

A characteristic feature of fresh brain tissue is a high initial rate of oxygen uptake usually considerably greater than that of the same weight of muscle. (Leg muscle was usually taken.) A fresh guinea-pig brain takes up oxygen at an initial rate of about 200 mm.^{3} per g. of tissue per 15 min. and this rate is kept at very nearly a constant level for over 2 hours after which it begins to fall. Usually this rate has fallen to between two-thirds and half the initial value in 3 hours (see Figs. 1, 2, 4). There is very little difference between the rates of autoxidation of the brains of well-fed and starved animals. The total volume of oxygen taken up by 1 g. of brain tissue of the guinea-pig in 3 hours is approximately 2000 mm.^{3} Values of the oxygen uptake by the brain tissue of various animals are shown in Table I.

Although the autoxidation of brain tissue is always of a high order, the actual value is not constant, under our experimental conditions, for a particular animal species and it is impossible to use this value, therefore, as an index to the condition of a tissue. The order of variability is shown in the following figures which give the O_2 uptake in 90 min. by 0·5 g. fresh rabbit brain: 584, 431, 599, 685, 678 mm.^{3}

It was felt however that the extra oxygen uptakes due to the addition to the brain of various substrates or metabolites might prove to be constant and offer useful indices for judging the normality or abnormality of a tissue.

Glucose and sodium lactate (at concentrations of $M/30$) were first tested but these proved to be quite unsuitable under the given experimental conditions. The extra oxygen uptakes by these substances after 90 or 120 min.

[1] Ringer solution: NaCl 0·9 %, KCl 0·025 %, $CaCl_2$ 0·03 %, $NaHCO_3$ 0·015 %.

Table I.

The oxygen uptakes recorded in this table, which are representative of a large number of experiments, are mm.^{3} obtained after 90 min. at 37°, 0·5 g. brain tissue being used. Mixtures of several brains were made with the very small animals. Grey matter of the cerebral cortex was used in the cases of the cat, sheep and man.

Animal	Autoxidation	O_2 uptake in presence of succinate (0·05 *M*)	O_2 uptake in presence of *p*-phenylenediamine (21 mg.)
Mouse*	696	1300	2212
	480	1450	1850
Rat	788	1455	1635
	680	1680	1428
Guinea-pig	517	1438	1562
	645	1188	1480
Rabbit	599	1392	1560
	685	1320	1525
Cat	422	1040	1560
Fowl	766	1367	1598
	680	1480	1342
Pigeon*	844	2260	2310
Man	444	710	1402
Dog	390	680	1325
Sheep	540	960	—

* In these cases 0·25 g. brain was used and the figures are calculated for 0·5 g.

were small and irregular, and it was sometimes observed that the addition of glucose inhibited the initial rate of autoxidation of brain. It seems most likely that the irregularities were due to variations in the content of lactic acid initially in the brain. Ashford and Holmes [1931] have shown that the extra oxygen uptake due to lactate by brain in the presence of air does not become marked until the autoxidation has fallen away from its initial linear rate.

As is well known, a number of substrates will act as hydrogen donators in the presence of brain and these, on addition to tissue, will bring about an extra uptake of oxygen. Chief amongst these is succinate, which is oxidised freely and which is converted quantitatively into *l*-malate [Quastel and Wheatley, 1931, 1]. Now it has been found in studies on the connection between cell structure and cell activity [Penrose and Quastel, 1930] that the mobilisation of active hydrogen by the cell depends greatly on its integrity. Using bacteria it was found that disintegration of the cell structure resulted in a great diminution in the oxygen uptake induced by added hydrogen donators. The oxidation due to succinate was reduced over 90 %. Hence it was to be anticipated that any profound change in cell structure would be reflected in a lowered oxygen uptake by the brain in presence of hydrogen donators.

As sodium succinate is powerfully activated by normal brain tissue, it was chosen as a suitable hydrogen donator for the study of brain on these lines.

Another substance upon which attention was turned was *p*-phenylenediamine. Keilin [1929] has made it appear very probable that the *p*-phenylenediamine oxidase plays an essential part in the normal respiratory activity of

the cell and that the oxidase is identical with Warburg's "respiratory ferment." It has been shown [Penrose and Quastel, 1930] that the activity of the oxidase is independent of the integrity of cell structure, and that narcotics do not affect its action in the way that dehydrogenase activity is affected (Keilin). The oxidase, indeed, appears to belong to that category of substances to which cytochrome is related. The rate of oxidation of *p*-phenylenediamine depends on a tissue oxidase quite distinct from that responsible for the activation of the usual hydrogen donators and hence should give another index for judging the normality or abnormality of a particular tissue. Brain tissue oxidises *p*-phenylenediamine with great rapidity [*cf.* Holmes, 1930], grey matter being approximately four times as active as white.

Values of the oxygen uptakes found with the brain tissue of various animals in the presence of sodium succinate and neutralised *p*-phenylenediamine are recorded in Table I. These are typical of a large number of results.

It is noteworthy that the smallest animals give the largest velocities of oxidation of *p*-phenylenediamine. Mouse brain is much more effective than that of guinea-pig or rabbit, whilst the grey matter of cat, dog or man does not oxidise the diamine at a greater rate than does the whole brain of rabbit or guinea-pig. There appears to be an inverse relationship between the size of the animal (or its brain) and the ability of the brain to oxidise *p*-phenylenediamine. Such a relationship was also noticed by Vernon [1911, 1912] when studying the intensity of the indophenol reaction of various tissues. This relationship does not appear to extend to muscle. The muscle of mice, for instance, oxidises *p*-phenylenediamine at a slower rate than that of the guinea-pig. As a general rule the muscle is less active than the brain of the same animal in oxidising *p*-phenylenediamine. For instance whereas 0·5 g. rabbit brain took up 1560 mm.^{3} O_2 in 90 min. in presence of 21 mg. of *p*-phenylenediamine, 0·5 g. rabbit leg muscle took up 990 mm.^{3} O_2 under the same conditions. This diminished activity of muscle compared with brain is shown also in the oxidation of succinate. The extra O_2 uptake due to succinate (0·05 *M*) by 0·5 g. mouse brain was 604 mm.^{3}; the extra O_2 uptake under similar circumstances by 0·5 g. mouse leg muscle was 328 mm.^{3}

Of all tissues studied human grey matter showed the least activity towards succinate. Figures obtained with human material are open to the criticism that the brain is several hours old before examination can be made. It may be shown however that succinate oxidation by brain suffers no diminution on storage for 24 hours—whilst that of muscle diminishes considerably. Table II records values found for the autoxidation and for the oxidation of succinate with fresh rabbit brain and muscle and for oxidations with the same tissues 24 hours old (stored at 0°). The low oxygen uptakes found consistently with human material in presence of succinate appear to be characteristic of human tissue and are not due to the lapse of time between death and examination of the brain.

The values recorded in Table I though fairly descriptive of the activities

Table II.

	O_2 uptake in mm.^{3} in 1 hr.	O_2 uptake in mm.^{3} in presence of succinate in 1 hr.	Extra O_2 uptake in mm.^{3} in 1 hr.
Brain	539	1150	611
,, 24 hrs. old	410	1135	725
	(2 hrs.)	(2 hrs.)	(2 hrs.)
Muscle	124	870	746
,, 24 hrs. old	45	502	457

of the brain tissue of various animals fail on the whole to give the information which it was the object of the investigation to discover. Reliable and consistent values were required which could be used as indices of the normal condition of brain tissue. The values found, though hovering near a mean value were not sufficiently consistent for the purposes required. It became clear that the method would have to be abandoned unless a modification of the experimental technique was adopted which would lead to consistent and reproducible results.

The value which was found to be most subject to variation was the rate of autoxidation, pointing to the presence of variable amounts of lactic acid and other substances in the brain of freshly killed animals. The question which arose was whether the extra oxygen uptake due to the addition of a metabolite such as succinate would be independent of the rate of autoxidation. If succinate, of which the brain initially contains but a trace, is oxidised independently of other material in the tissue, the extra O_2 uptake due to this substance would be a constant descriptive of the tissue under investigation. It has been found, however, that the extra oxygen uptake due to succinate varies inversely with the rate of autoxidation and it is now certain that the rate of oxidation of succinate is greatly dependent on the presence of other oxidisable material in the brain. This is shown as follows.

Oxidation of succinate.

0·5 g. of chopped guinea-pig brain was placed in each of the right-hand vessels of three Barcroft manometers. In the first was placed 1 cc. *M*/5 phosphate buffer p_H 7·4, 2 cc. saline and 0·5 cc. of 0·4 *M* sodium succinate, in the second was placed the same with the exception of the succinate and in the third was placed the same with 0·5 cc. of 0·4 *M* NaCl replacing the succinate. After attainment of temperature equilibrium the taps of the apparatus were closed and the rates of oxygen uptake in the three manometers read. Two hours later the contents of the first vessel were examined polarimetrically, the volume being made up to 5 cc. and 1 cc. glacial acetic acid and 10 cc. 14·2 % ammonium molybdate solution being added. This was filtered and the filtrate examined in a 1 dm. tube using the mercury green line. [For details of this method of estimation of *l*-malic acid, see Quastel, 1931.] One hour later—*i.e.* after autoxidation had proceeded in the remaining Barcroft manometers

for 3 hours—0·5 cc. of 0·4 *M* sodium succinate solution was added to the second vessel, the rate of oxygen uptake having dropped to about 2/3 its initial value. The rates of oxidation in the two vessels were measured for a further 2 hours, after which the contents of the two vessels were examined polarimetrically by the method described. The following were the figures obtained:

First vessel (succinate added initially)	
Oxygen uptake in 2 hrs.	1690 mm.³
Rotation found	+0·44°
Second vessel (succinate added after 3 hrs. autoxidation)	
Oxygen uptake in the second period of 2 hrs. ...	1667 mm.³
Rotation found	+0·68°
Third vessel (no succinate added at any time)	
Oxygen uptake in first period of 2 hrs.	745 mm.³
Oxygen uptake in second period of 2 hrs.	250 mm.³
Rotation found	0·00°
Extra O_2 uptake due to succinate in first vessel ...	945 mm.³
Extra O_2 uptake due to succinate in second vessel ...	1417 mm.³

It is clear from the figures quoted that the extra oxygen uptake due to addition of succinate varies inversely with the content of oxidisable material in the brain and that the *l*-malic acid formed (as shown by the rotation) varies in the same way. Now it is known that fumarate is transformed into *l*-malate in the presence of brain with great rapidity [see Quastel, 1931], and hence the rotations shown represent equilibrium values between the fumarate and *l*-malate. It is easy to show that the uptake of 1000 mm.³ of O_2 at 37° in the oxidation of succinate corresponds to the formation of *l*-malate whose rotation, measured in the manner described above, would be +0·48°. Hence the rotation corresponding to the extra O_2 uptake of 945 mm.³ would be +0·45° and that corresponding to an uptake of 1417 mm.³ would be +0·67°. The figures found were +0·44° and +0·68° respectively, showing that the extra oxygen uptakes are almost exactly equivalent to the *l*-malic acid formed. Thus the extra O_2 uptake is entirely concerned with the oxidation of succinate and it is evident that the oxidation of succinate by the brain is diminished by the presence of other oxidisable material in the brain.

It was decided in view of this result to modify the original experimental technique by allowing the brain tissue to autoxidise for 3 hours, *i.e.* till the rate of O_2 uptake had fallen to between 2/3 and 1/2 of its initial uptake, before adding substrates or metabolites. With this modified technique constant and reliable results have been obtained.

Oxidation of the sugars.

The addition of glucose at a concentration of 0·025 % to a brain tissue which has been greatly depleted of its autoxidisable materials results in a quick recovery in the rate of oxidation to a level nearly that of the initial rate (Curve A, Fig. 1). This new level remains constant for a lengthy period. The

rate of oxygen uptake after addition of glucose is directly proportional to the amount of tissue present. This is shown by the following figures:

Amount of tissue to which glucose has been added g.	O_2 uptake in 2 hrs. mm.^{3}	O_2 uptake. / Amount of tissue mm.^{3}
0·5	868	1736
0·3	568	1866
0·2	366	1830
0·1	166	1660

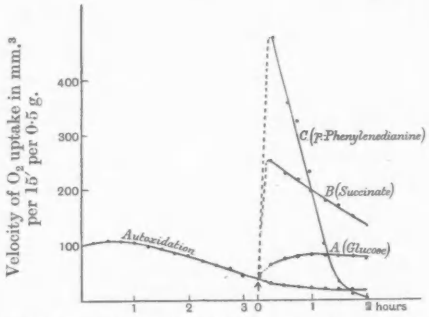


Fig. 1. Effects of addition of glucose (0·025 %) sodium succinate (0·05 *M*) and *p*-phenylenediamine (21 mg.) on rate of O_2 uptake of guinea-pig brain.

It was found in agreement with Loebel's results [1925] that the addition of fructose and mannose results in an increased rate of oxygen uptake. Glycogen and galactose have very little effect on the restoration of the rate of O_2 uptake whilst mannitol and gluconic acid are inert under the given experimental conditions. The pentoses, arabinose and xylose, were also investigated and found to be inert. The results are shown in Table III.

Table III.

O_2 uptake in mm.^{3} by 0·5 g. brain tissue (guinea-pig) largely depleted of autoxidisable material by autoxidation for 3 hours prior to addition of substrates. The sugars were all added in saline solution.

Time (min.)	30	60	90	120
Saline	96	170	225	269
Glucose (0·06 %)	165	396	647	893
Fructose (0·06 %)	200	400	601	784
Mannose (0·025 %)	160	332	516	662
Galactose (0·06 %)	148	250	319	375
Mannitol (0·06 %)	95	176	245	299
Arabinose (0·06 %)	74	135	186	232
Xylose (0·06 %)	84	157	228	289
Glycogen (0·06 %)	99	201	292	375
Sodium gluconate (0·025 %)	82	148	205	260
Sodium succinate (0·05 *M*)	493	927	1316	1620
Sodium fumarate (0·0125 *M*)	99	183	254	302
Sodium lactate (0·125 *M*)	176	401	628	848
Sodium lactate (0·0025 *M*)	139	265	385	490
Sodium pyruvate (0·0125 *M*)	192	395	601	802
Sodium glutamate (0·05 *M*)	144	291	436	559
Serum (0·5 cc.)	160	321	472	630

Oxidation of lactic and pyruvic acids.

As is now known from the work of Loebel [1925] and Ashford and Holmes [1931], the addition of sodium lactate and sodium pyruvate to the brain results in a prolongation of the steady rate of oxygen uptake. The addition of lactate at so low a concentration as 0·0025 *M* to brain tissue greatly depleted of oxidisable material results in a restoration of the linear uptake of O_2 by the brain. This is shown in Fig. 2, which gives the results of an experiment carried on for 9 hours. The rate of autoxidation had fallen to 2/3 the initial level in 3 hours, when 0·9 mg. lactic acid (as sodium lactate) was added to the tissue. It will be seen that the rate of oxidation in presence of the lactate falls off slowly in marked contrast to that in the presence of succinate where

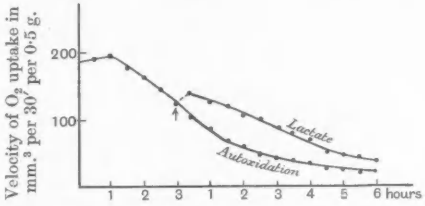


Fig. 2. Effect of addition of 0·9 mg. lactic acid on rate of oxidation of guinea-pig brain.

the rate falls off quickly (compare Curve B, Fig. 1, the concentration of succinate initially being 0·05 *M*). The volume of extra oxygen consumed, after oxidation had proceeded in presence of the added lactate (0·9 mg.) for 6 hours was 444 mm.³ and oxidation was still taking place at a fairly high rate. The theoretical oxygen uptake for the complete oxidation of this amount of lactate is 768 mm.³ at 37°. There seems little doubt that the theoretical oxygen uptake would have taken place had it been possible to carry out the experiment for a sufficiently long time.

This point is of some importance in connection with the statement of Ashford and Holmes [1931] that less oxygen is taken up by brain tissue than can be accounted for by the disappearance of lactate. It seems clear from a study of the rate of oxidation that even if lactic acid is converted by the brain into some substance which is not estimated as lactic acid it is eventually entirely oxidised. Reference will be made to this later.

It has been suggested [Toenissen and Brinkman, 1930] that the path of oxidation of lactic acid lies through pyruvic acid, αδ-diketoadipic acid and thence through succinic and fumaric acids. Now experiment shows that fumaric acid is exceedingly slowly oxidised by the brain (see Table III), though *l*-malic acid is formed from it with great rapidity. The very slow rate of oxidation of fumaric acid should bring about an accumulation of this substance (and, therefore of *l*-malic acid) among the products of oxidation of lactic acid—for both lactic and pyruvic acids at small concentrations are

oxidised at a higher rate than relatively large concentrations of fumaric acid. Ashford and Holmes [1931] could find no evidence of accumulation of a ketonic acid. Polarimetric examination of the products of oxidation of lactic acid in the presence of brain showed no trace of the formation of *l*-malic acid and it must be concluded that the evidence does not support the hypothesis that lactic acid (or the major part of it) is oxidised through succinic acid.

The addition of lactate or pyruvate to brain tissue which has been partially depleted of its autoxidisable material restores the rate of oxidation to a level equal to or a little higher than that brought about by the addition of an equivalent quantity of glucose. This result is in agreement with the hypothesis, for which Holmes has produced evidence, that glucose must be converted into lactic acid for its oxidation to occur in presence of the brain. The conclusion, too, is supported by the fact that the addition of glucose to brain tissue consuming lactate does not increase the rate of oxidation. This is shown by the following figures:

	mm.³
O_2 uptake in 2 hrs. by 0·5 g. brain (guinea-pig) partially depleted of oxidisable material by autoxidation previously for 3 hrs.	236
O_2 uptake in 2 hrs. in the presence of lactate (0·025 *M*) by 0·5 g. of the same tissue	756
O_2 uptake in 2 hrs. in the presence of glucose (0·0125 *M*)...	589
O_2 uptake in 2 hrs. in the presence of glucose (0·0125 *M*) and lactate (0·025 *M*)	628

It has been shown by Loebel [1925] and it is indicated in the values recorded in Table III that fructose is oxidised by brain tissue at a rate approaching that at which glucose is attacked. Yet according to Loebel fructose gives rise to little or no lactic acid in presence of brain. The conclusion would be that fructose is oxidised on lines different from those of glucose oxidation.

Krebs [1931] has shown that the addition of iodoacetic acid to the brain prevents the oxidation of glucose, presumably by attacking the mechanism by which glycolysis is effected. It was thought possible that iodoacetic acid would not affect the oxidation of fructose if the production of lactic acid is not an important path of the breakdown of this sugar. The following experiments were therefore undertaken.

Action of iodoacetic acid.

Sodium iodoacetate solution was added to fresh brain tissue to give a final concentration of *M*/4000. Autoxidation was allowed to take place for 3 hours, after which the effects of the addition of glucose, lactate *etc.* were determined. The results are noted in Table IV.

It will be seen that the oxidations of glucose, fructose and mannose are greatly inhibited (over 90 %). The oxidations of lactate and pyruvate are also affected but not to the same extent.

Holmes [1930] has shown that glucose oxidation proceeds independently of a phosphoric acid ester stage. Iodoacetic acid inhibits, presumably, a direct glycolysis of glucose. It is conceivable that fructose oxidation proceeds through a phosphoric ester stage and that iodoacetic acid inhibits the forma-

Table IV.

O_2 uptakes by 0·5 g. guinea-pig brain tissue in the presence of various substrates after treatment with *M*/4000 iodoacetic acid and autoxidation for 3 hrs.

Time (min.)	30	60	90	120
Saline	41	77	105	126
Sodium lactate (0·0125 *M*)	104	213	317	408
Sodium pyruvate (0·0125 *M*)	105	211	316	410
Glucose (0·025 %)	55	100	132	171
Fructose (0·025 %)	58	104	140	168
Mannose (0·025 %)	61	105	138	168
Sodium glutamate (0·05 *M*)	87	176	255	324
Serum (0·5 cc.)	114	195	265	317
Sodium succinate (0·05 *M*)	498	922	1280	1578

tion or breakdown of the ester in the manner suggested by Lohmann [1931]. On the other hand hexosediphosphoric acid is only attacked very slowly by brain tissue and a sample of synthetic 6-hexosemonophosphoric acid[1] proved to be inert, as shown by the following figures.

	mm.³
O_2 uptake by rat brain in 2 hrs.	244
O_2 uptake by rat brain in presence of sodium hexosediphosphate (0·25 %) ...	302
O_2 uptake by rat brain in presence of sodium 6-hexosemonophosphate (0·06 %)	240
O_2 uptake by rat brain in presence of glucose (0·06 %)...	583

The brain tissue was partially depleted of oxidisable material by prior oxidation for 3 hours.

The possibility remains that fructose gives rise to lactic acid at a very slow rate but sufficient to provide lactic acid in a concentration which would give the observed rate of oxidation. The question must remain in abeyance until more is known of the mechanism of fructose breakdown in presence of brain. In the meantime, the action of iodoacetic acid on the oxidation of glucose does not constitute, in view of its effect on fructose, evidence that glucose necessarily passes through lactic acid for its oxidation to take place. There is indeed an entire lack of evidence as yet to show how far iodoacetic acid affects the initial activation of carbohydrates by brain tissue, *i.e.* preliminary to subsequent metabolic changes. The same criticism applies to the action of sodium fluoride which inhibits glycolysis and glucose oxidation to a greater extent than lactate oxidation [Holmes, 1930; Ashford and Holmes, 1929]. It applies also to the action of oxalate which has a marked toxic action on the oxidation of glucose by brain; indeed oxalate at a concentration of 0·025 *M* is so toxic that it inhibits the oxidation of lactate.

Relative effects of phosphate buffer and Ringer solution.

It has been shown that lactic acid production from glucose proceeds independently of added phosphate ions and that it proceeds actively in a bicarbonate buffer [Ashford and Holmes, 1929]. Experimental results shown in Fig. 3 indicate that if brain tissue is depleted of much of its oxidisable material

[1] We are much indebted to Baeyer Products Ltd. for a gift of calcium hexosediphosphate and to Dr P. A. Levene for a sample of 6-hexosemonophosphate.

in the presence of Ringer solution the subsequent addition of glucose gives rise to a diminished extra oxygen uptake. The addition of phosphate buffer solution at the same time as the glucose brings about, however, the usual increased rate of oxygen uptake by the brain.

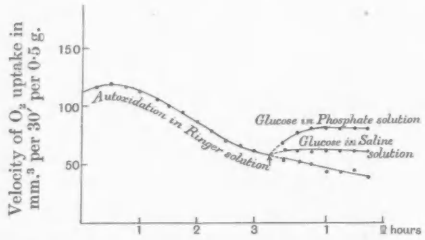


Fig. 3. Effects of addition of glucose (0·025 %) in phosphate buffer solution p_H 7·4 and in saline respectively on rate of O_2 uptake by guinea-pig brain suspended in Ringer solution.

It is observable in all experiments where glucose is added to brain tissue suspended in Ringer solution that acidity develops, a p_H of 6·8 being obtained. Lactate in phosphate buffer at this p_H is only very slowly oxidised, as the following results indicate: the brain tissue had been allowed to autoxidise in the usual way for 3 hours prior to addition of lactate.

	mm.^{3}
Total O_2 uptake by lactate (0·025 *M*) in presence of 0·5 g. brain tissue suspended in phosphate buffer at p_H 6·8	218
Ditto but with tissue suspended in phosphate buffer at p_H 7·4	450

It is probable therefore that the lack of oxidation of glucose in Ringer solution by brain is due to the development of a relatively high hydrogen ion concentration.

Oxidation of glutamic acid.

Glutamic acid (0·05 *M*) is oxidised at a comparatively slow rate by either fresh brain tissue or tissue which has been depleted of oxidisable material by previous oxidation for 3 hours. The following figures are typical for guinea-pig brain. (Glutamic acid was always neutralised with sodium hydroxide.)

	mm.^{3}
O_2 uptake of fresh brain (0·5 g.) in 1½ hours	770
O_2 uptake of fresh brain with glutamic acid (0·05 *M*) added initially (in 1½ hrs.)	942
O_2 uptake by brain in 1½ hours, after 3 hours prior autoxidation	200
O_2 uptake by brain in 1½ hours, with the glutamic acid added subsequent to the 3 hours autoxidation	388

Polarimetric examination of the products of oxidation of glutamic acid by brain tissue showed no indication of *l*-malic acid formation.

Iodoacetic acid (*M*/4000) had but little effect on the oxidation by brain of the amino-acid (see Table IV).

The action of serum.

It was interesting to observe the effects of the addition of serum to brain tissue which had been depleted of much of its oxidisable material. An immediate rise in the rate of oxidation took place. Values representative of the increased O_2 uptake due to serum are shown in Table III. The effect is of the same order as that of glucose or lactate and is due doubtless partly to the presence of these substances in the serum. A brain treated with iodoacetic acid showed an increased O_2 uptake in presence of serum (Table IV). Serum, as control experiments showed, does not interfere with the action of iodoacetic acid.

Oxidation of succinate and p-*phenylenediamine.*

Table V records typical values indicating the oxygen uptake by brain tissue of various animals in the presence of glucose, sodium succinate and *p*-phenylenediamine—the brain tissue having been allowed to take up oxygen

Table V.

O_2 uptake in mm.^{3} in 1 hour by 0·5 g. brain tissue, which had been allowed previously to take up oxygen for 3 hours, in the presence of glucose (0·025 %), sodium succinate (0·05 *M*) and neutralised *p*-phenylenediamine hydrochloride (21 mg.).

	Saline (autoxidation)	Glucose	Sodium succinate	*p*-Phenylene-diamine
Rabbit	162	229	790	1315
Mouse	154	345	1122	1534
Guinea-pig	154	287	870	1406
Bullock (grey matter)	179	250	790	1190

for 3 hours prior to the addition of substrates—and Curves A, B and C, Fig. 1, show the great differences in rates of oxygen uptake induced by them. The modified technique results in values being obtained which are remarkably uniform. The following figures show the extra O_2 uptake in $1\frac{1}{2}$ hours by 0·5 g. guinea-pig brain tissue in the presence of sodium succinate (0·05 *M*), using the new technique; 1066, 1066, 1055, 993, 989 mm.^{3} Considering that guinea-pigs from various sources were used and that the total experimental error involved in the determination of O_2 uptake is of the order of 5 %, the uniformity of the results speaks well for the utility of the method. Human material has not yet been investigated with the modified technique.

Table V shows the high oxidising activity of mouse brain towards glucose and succinate as compared with the brain of guinea-pig or rabbit. It is found that the initial rate of oxidation of glucose by mouse brain is more than double that found with guinea-pig or rabbit. The oxidation of succinate by mouse brain proceeds at a velocity 50 % greater than that by rabbit brain and the velocity of oxidation of *p*-phenylenediamine is also greater as was found in the preliminary experiments recorded in Table I. The value of the modification of the original technique is seen on comparing the results of Table I and Table V. In the early experiments owing to the high autoxidation of

brain it was impossible to obtain a fair estimate of the rate of attack of succinate—and experiments with glucose were out of the question. The new results confirm the previous conclusion that the rate of oxidation of various metabolites by brain tissue is inversely proportional to the size of the animal.

l-Malic acid production from succinate in the presence of lactate.

It has already been shown that the extra oxygen uptake and the *l*-malate production consequent on addition of succinate to a fresh brain are less than those obtained when succinate is added to a brain greatly depleted of its autoxidisable material. The question arises as to whether this is due to interference with succinate oxidation by some normal constituent of brain tissue. Since it is known that a part of the normal oxygen uptake of brain is due to lactate, experiments were devised to ascertain whether

(*a*) a mixture of succinate and lactate was oxidised at a rate higher than either of the two individually,

(*b*) *l*-malate production from succinate was affected by the presence of lactate.

Results are recorded in Fig. 4 which shows the effects of adding succinate, lactate and a mixture of succinate and lactate to brain tissue which had been

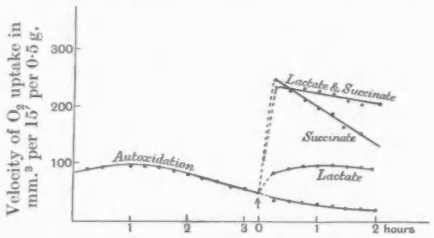


Fig. 4. Effects of addition of sodium lactate (0·0125 *M*), sodium succinate (0·05 *M*) and a mixture of these on rate of O_2 uptake of guinea-pig brain.

allowed to take up oxygen for 3 hours prior to addition of the substrates. It is clear that the velocity of O_2 uptake is not greater with the mixture of succinate and lactate than with succinate alone—but the high level in the rate of oxidation is retained for a longer period with the mixture than with the individual substrates. Polarimetric examination of the products of oxidation showed the following results:

Rotation found with lactate alone	0·00°
Rotation found with succinate alone	+0·66°
Rotation found with lactate + succinate	+0·48°

The oxidation of succinate is diminished by the presence of lactate, the amount of diminution being almost exactly equivalent to the amount of oxidation for which lactate alone is responsible. This is shown by the following figures which are typical of a number of experiments. They are the values

found after 2 hours' oxidation with 0·5 g. guinea-pig brain tissue which had been allowed to oxidise for 3 hours prior to addition of substrates. The sodium salts of lactic and succinic acids were used at concentrations of 0·0125 *M* and 0·05 *M* respectively.

	mm.³
O_2 uptake by tissue alone	231
O_2 uptake by tissue + lactate	756
O_2 uptake by tissue + succinate	1580
O_2 uptake by tissue + lactate + succinate	1752
Extra O_2 uptake due to lactate	525
Extra O_2 uptake due to succinate	1347
Extra O_2 uptake due to lactate + succinate ...	1521

Polarimetric examination:

Rotation found with succinate alone ... ... +0·66°

Rotation calculated for succinate + lactate $= (1521 - 525) \times \frac{0{\cdot}48°}{1000} = 0{\cdot}478°$

Rotation found for succinate and lactate ... ... +0·48°

The calculation of rotation is made on the basis that the rate of oxidation of succinate (in the mixture) is that of the mixture *minus* the rate of oxidation due to the lactate alone (this, it is assumed, being unaffected by the presence of succinate). The calculated and observed values of *l*-malate production in the mixture of succinate and lactate are identical within the limits of experimental error. Hence the oxidation of succinate by brain is "spared" by the presence of lactate to an extent equivalent to the amount of oxidation for which lactate alone is responsible.

This finding has an important bearing on Ashford and Holmes' investigation [1931] of the "Meyerhof quotient" in brain. These authors have found that more lactic acid disappears in the presence of brain than can be accounted for by the extra oxygen uptake. This could be explained by a lessened oxidation of proteins or lipoids in the presence of lactate, but Ashford and Holmes concluded that the sparing action was improbable because (unoxidised) breakdown products of protein or lipoid could not be found in a brain tissue consuming added lactate. The results given above, however, show definitely that lactate "spares" the oxidation of succinate and since it is unlikely that this sparing action is unique and that it only affects succinate there is every probability that the oxidation of normal constituents of the brain is spared by lactate. This is all the more significant when it is realised that the total oxygen uptake of brain is more than double that required to oxidise the entire quantity of lactic acid initially present.

Let us now consider the mechanism of this sparing action.

Mechanism of the sparing action of lactate on succinate oxidation.

Three suggestions offer themselves as explanations for this sparing action of lactate and these will be examined in turn.

A. Lactate in the presence of brain tissue and the succinate-fumarate system may not be directly oxidised but may reduce fumarate to succinate at a rate comparable with the formation of fumarate from succinate by oxida-

tion. Thus fumarate would not accumulate in the presence of lactate and a sparing action by the lactate would be evinced.

This suggestion can be experimentally tested. If lactate reduces fumarate at a speed at all comparable with succinate oxidation it is clear that the addition of lactate to fumarate in the presence of brain tissue will reduce the yield of *l*-malate by an amount proportional to the succinate formed. Accordingly 0·5 g. guinea-pig brain tissue was allowed to autoxidise in the usual way for 3 hours in the vessels of two Barcroft manometers. Sodium fumarate solution (0·025 *M*) was added to one vessel and to the other was added a mixture of sodium fumarate (0·025 *M*) and sodium lactate (0·025 *M*). The air in the manometer vessels was displaced by nitrogen and under these anaerobic conditions the tissues were allowed to remain for 2 hours. Polarimetric examinations of the contents of the two vessels were then made and it was found that the rotation (0·29°) was identical in both cases. No measurable reduction of fumarate by lactate had taken place and the possibility of lactate sparing by means of fumarate reduction must be dismissed.

B. Lactate may become adsorbed at the succinic acid oxidising enzyme, a competition between lactate and succinate taking place for the same oxidising catalyst. This suggestion was experimentally investigated in the following way.

Assuming that the sparing of succinate takes place by preferential adsorption of, or a competition by, lactate for the same oxidising enzyme, it is clear that if brain tissue can be so treated that lactate oxidation is inhibited whilst that of succinate is unimpaired, the addition of lactate to such a tissue should reduce the velocity of oxidation due to succinate. The amount of reduction would depend on the amount of adsorption of, or competition by, lactate for the succinate oxidising enzyme.

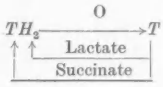
It is necessary at this juncture to anticipate certain results which will be published in full in a subsequent paper. They are to the effect that narcotics, in low concentrations, inhibit the oxidation by the brain of glucose, lactate or pyruvate and that they have no effect on the oxidation of succinate.

For the purposes of this experiment chloretone (0·12 %) was used. Brain (guinea-pig) tissue was allowed to autoxidise in the usual way in the presence of chloretone for 3 hours. After this interval the rates of O_2 uptake after addition of lactate (0·025 *M*), succinate (0·025 *M*), and a mixture of lactate (0·025 *M*) and succinate (0·025 *M*) were measured. The following were the results obtained (2 hours being the experimental period):

	mm.³
O_2 uptake by 0·5 g. tissue alone	236
O_2 uptake by tissue + lactate	298
O_2 uptake by tissue + succinate	1740
O_2 uptake by tissue + lactate + succinate	1750
Polarimetric examination:	
Rotation due to tissue + lactate	0·00°
Rotation due to tissue + succinate	+0·72°
Rotation due to tissue + lactate + succinate	+0·70°

It will be observed that with a chloretone-treated brain the presence of lactate does not appreciably affect the oxidation of succinate, either by reducing the oxygen uptake or the yield of *l*-malic acid. This result dismisses the possibility that lactate is adsorbed at or competes with succinate for the enyzme oxidising the latter.

C. The oxidation of both succinate and lactate may necessitate the intermediate action of a carrier, in the manner shown in the diagram. Assuming



this to be the case, the rate of oxidation of lactate or succinate or that of a mixture of the two will be determined by the rate of oxidation of the reduced form of the carrier TH_2 and by the rate of reduction of the oxidised form T. Lactate and succinate will compete for reduction of T, since only a limited quantity of this is available. Preferential attack of T by lactate may then simply depend on an easier access of T to the site of activation of the lactate molecule.

It is possible to test this view by examining the action of lactate on the oxidation of succinate when this is present at low concentrations.

According to the view that the oxidation of a hydrogen donator by the cell depends upon the reduction of T to TH_2 and the subsequent oxidation of TH_2 to T, it is evident that the rate of oxidation of any donator will depend upon its rate of production of TH_2. A given concentration of a donator will bring about the production of a certain concentration of TH_2 which will determine the velocity of oxidation. Let this concentration, for a given concentration of lactate, be αc where c represents the total concentration of TH_2 available; similarly let the concentration for a certain quantity of succinate be βc. Then the velocity of oxidation due to the lactate will be $\kappa\alpha c$, that for the succinate will be $\kappa\beta c$, and that for the two together will be $\kappa\alpha c + \kappa\beta c$ (κ being a constant). Now keeping the concentration of lactate constant, let us increase the concentration of succinate. The limiting velocity of oxygen uptake by the cell is obviously κc; hence when the succinate concentration increases to the extent that $\beta = 1 - \alpha$, the limiting velocity of oxygen uptake of the cell has been achieved. A further increase in succinate concentration may be expected to involve a sharing of the total quantity of TH_2 between the succinate and lactate. By hypothesis, however, the availability of T is so much more favourable for lactate than for succinate, that such sharing will not take place, α remaining constant for the given concentration of lactate. Hence increase in succinate concentration cannot bring about a greater velocity of oxidation than $\kappa(1 - \alpha)c$, though in the absence of the lactate the velocity will become κc, owing to all the carrier being available.

Hence the sparing of oxidation of succinate by lactate will only take place

when the cell is working at its full capacity, the oxidation of lactate proceeding at a constant rate independent of the amount of succinate. With low concentrations of succinate, it is to be expected that much less "sparing" of oxidation by lactate will take place. Experiment confirms this:

	mm.³
O_2 uptake in 2 hrs. by 0·5 g. guinea-pig brain tissue after previous oxidation for 3 hrs.	231
O_2 uptake by the tissue in presence of 0·0125 *M* lactate	756
O_2 uptake by this tissue in presence of 0·0125 *M* sodium succinate	638
O_2 uptake by this tissue in presence of a mixture of 0·0125 *M* lactate and 0·0125 *M* succinate	1102

Polarimetric examination showed that the *l*-malic acid production with succinate alone corresponded to a rotation of + 0·20° and that with the mixture of lactate and succinate, to a rotation of + 0·17°.

The fact that cytochrome [Holmes, 1930] is abundant in the grey matter of brain makes it possible that this substance is identical with the TH_2–T system.

Ashford and Holmes [1931] do not lean to the view that lactate has any appreciable sparing action on the oxidation of tissue components, their conclusion being based on the fact that unoxidised hydrolysis products of tissue or lipoid were not found in brain tissue in which lactate was being oxidised. This argument does not seem to be flawless, for it may be conceived that oxidation of a saturated (? lipoid) molecule may proceed normally to an unsaturated form without an entire break-up of the molecule preliminary to oxidation and this oxidation may be spared by lactate in much the same way as succinate oxidation is spared.

Such sparing action could not account for all Ashford and Holmes's results, for, as they point out, under certain circumstances more lactic acid disappears than can be accounted for by the total oxygen uptake. On the other hand it is observed that the total oxygen uptakes obtained in the presence of concentrations of lactate which are small enough to be comparable with those present initially in the brain, are sufficient to account for the entire oxidation of lactate. Only with relatively high concentrations of lactate does a considerable discrepancy arise and it seems probable that here a product is formed which escapes oxidation and which cannot be estimated as lactic acid. Similar experiments with pyruvate would probably prove to be instructive. In the case of bacteria, pyruvate is transformed into a substance which seems to regenerate pyruvate on subsequent treatment with alkali [Quastel and Wooldridge, 1929]. Whether this occurs with brain tissue is a matter for further investigation.

SUMMARY.

1. The rates of oxygen uptake by brain tissue of various animals and the effects upon these of the addition of a number of substrates have been studied. Of all tissues investigated human grey matter shows the least activity towards the oxidation of succinate.

2. A modification of the usual technique is described whereby brain tissue is allowed to become greatly depleted of its oxidisable material before substrates are added to it. Using this method constant and reproducible results are obtained for the rates of oxidation of glucose, sodium succinate, *p*-phenylenediamine *etc.* in the presence of brain tissue of various animals.

3. The rate of oxidation of added substrates to the brain varies inversely with the size of the animal. This generalisation does not apply to the oxidations of muscle.

4. Glucose, sodium lactate and sodium pyruvate at equivalent concentrations are oxidised at approximately the same rate by brain tissue. Glucose is not appreciably oxidised by brain in Ringer solution, this probably being due to development of a high hydrogen ion concentration which retards the oxidation of lactate. The addition of phosphate buffer to Ringer solution restores the rate of oxidation due to glucose. Lactate is probably completely oxidised by brain. The addition of serum increases the oxygen uptake due to brain.

5. Iodoacetic acid (*M*/4000) inhibits the oxidation by brain of glucose, fructose and mannose. This result is discussed in view of the statement that fructose does not give rise to lactic acid in presence of brain. Iodoacetic acid only partially inhibits the oxidation of lactate, pyruvate and glutamate. It has no effect on succinate oxidation.

6. The oxidation of succinate by brain tissue varies with its content of oxidisable material.

7. The presence of lactate "spares" the oxidation of succinate by brain. In a mixture of lactate and succinate, the oxidation of the latter (as measured by *l*-malate production) is inhibited by an amount exactly equivalent to the oxidation for which the lactate alone is responsible.

8. The mechanism of the sparing action due to lactate on succinate oxidation is discussed. It is shown that it cannot be due to reduction of fumarate by lactate, nor to competition by lactate with succinate for the enzyme oxidising the latter. It is concluded that the action is due to competition of lactate with succinate for the oxidised form of a carrier (? cytochrome). The significance of the sparing action of lactate is discussed.

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XC. BLOOD-UREA CHANGES IN ABNORMAL MENTAL STATES AFTER THE ADMINISTRATION OF AMINO-ACID.

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A study of the changes occurring in blood-amino-acid and urea following the ingestion of protein or glycine has been made, in order to determine whether any disturbance of nitrogen metabolism occurs in abnormal mental states. This work was suggested by a consideration of the disorders of carbohydrate metabolism found in psychotic subjects. There is general agreement that, while the fasting blood-sugar levels are within normal limits, in certain cases there is a lowered sugar tolerance and it has been recently established by McCowan and Quastel [1931] that a sustained hyperglycaemia is intimately associated with the affective state and varies *pari passu* with the degree of emotional tension experienced by the subject. It was thought possible that some comparable deviation from the normal might occur during protein breakdown and we therefore investigated the fasting blood in a series of cases and noted the changes in the amino-acid and urea levels at stated intervals following the administration of gelatin or glycine.

Our results, which are incorporated in the following paper, show that, among psychotics, the changes in the urea levels tend to be of a large order as compared with the normal and that two types of curves exist which are distinguished by variations in the rate of rise and fall of urea. Further, an association has been found between the type of curve and the clinical state.

Many investigations of the nitrogenous constituents of the blood in abnormal mental states have been made [Bowman, 1922; Henry and Mangam, 1925; Looney, 1924; Reid, 1927] but we have been unable to find any reference to changes occurring following protein intake. There is general agreement that in all forms of psychosis the urea-nitrogen lies within normal limits and Bowman in a survey of 229 cases found this to be true also of the other nitrogenous constituents. His findings were confirmed by Henry and Mangam. Rappleye [1919] noted low blood-urea levels among psychotics and Northcote [1932], working in this laboratory, found several low urea levels in an investigation of recently admitted cases of all types. She considered this to be largely due to an inadequate diet prior to admission. In our series, a generally low

fasting level has been found in the melancholic group and in four cases the value was below 20 mg./100 cc.

Folin and Berglund [1922] investigated the changes in the blood of a normal man following the ingestion of 25 g. of glycine and found that the amino-acid concentration reached a maximum in the 2nd hour and the urea showed fluctuations of a small order up to the 5th hour. They do not state under what conditions the subject was placed during the experiment. Witts [1929] studied the changes in amino-acid and urea following the administration of glycine in both health and disease and found that the amino-acid curve was remarkably constant, a peak being reached in about 2 hours, followed by a steady fall to normal. The maximum value for urea was reached in 2–4 hours after the peak of the amino-nitrogen curve. On giving 12·5 g. of glycine to a fasting normal subject he observed an increase of 2 mg./100 cc. in 3 hours followed by a steady fall to the fasting level within 6 hours.

Methods and procedure.

The subjects investigated, including controls, were between the ages of 17 and 55; all were in fair physical condition and in none was there any evidence of visceral disease. In the female cases the test was carried out during the inter-menstrual period.

The subject received no food after 6.30 p.m. on the day prior to the test, was in bed by 9 p.m. and remained there the following day throughout the period of the experiment. After a 14-hour fast a sample of blood was taken and a meal of protein or amino-acid administered. Venesection was performed at hourly intervals for 5 hours.

In the early cases gelatin was used, 25 g. being dissolved in 300 cc. of water and flavoured with 20 cc. of fresh lemon juice; this set as a fairly stiff jelly which was found to be generally unpalatable and difficult to administer. For this reason and because of its simpler constitution glycine was substituted in later experiments, 10 g. being dissolved in 100 cc. of distilled water and flavoured with lemon juice. Glycine was used in all the control experiments.

Estimations of amino-acids and urea were made by the Folin [1922] colorimetric and the Van Slyke and Cullen [1914] urease methods respectively, duplicate analyses being made in all cases.

Results.

1. Amino-acid.

Amino-acid determinations were made on 17 consecutive cases of all types. The changes were small and varied but slightly from individual to individual. The total change was in no case greater than 3 mg./100 cc. and at the end of the 5-hour period the value had returned to within 10 % of the fasting level. The peak was reached generally in the 1st hour and never later than the 2nd, whilst the fall was gradual and uniform. The variations were so small

that no significance could be attached to the results and the determinations were discontinued in later experiments. No amino-acid estimations were carried out on controls.

2. Blood-urea: results in controls.

The subjects of the control experiments were four probationer nurses and a laboratory boy, all working at the Cardiff City Mental Hospital and examined under the same conditions as the patients. The blood-urea changes were of a small order, the maximum rise in no case exceeding 6·5 mg. per 100 cc. of blood (see Fig. 1), and in three cases being less than 1 mg. In every case

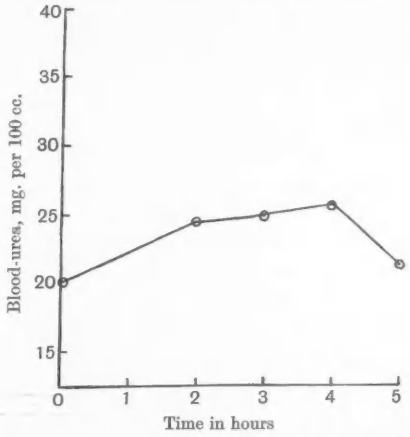

Fig. 1. Variations in blood-urea in a normal person after administration of 10 g. glycine.

there was evidence that the changes had reached their maximum before the end of the test period and a composite curve of this group as a whole is almost identical with that shown by Witts [1929], using the same quantity of glycine.

Results with patients.

Forty cases were examined and the results divide themselves into two distinct groups depending upon the rate of the changes in the urea levels. In both groups the changes tend to be of a large order.

Group 1 is characterised by a steady and uniform rise to a peak, occurring generally in the 3rd hour and followed by a rapid fall, the fasting level being reached in many cases by the 5th hour (*e.g.* Fig. 2). In addition the maximum changes, whether expressed in an absolute value of mg. of urea per 100 cc. of blood or as a percentage increase of the fasting level, were far in excess of the corresponding changes found in controls. The urea changes were about 14 mg. per 100 cc. of blood, or an increase of 50 % of the fasting level, a change which equals the changes found by Witts [1929] after the administration of double

the quantity of glycine to a normal subject. The variations between individuals were again marked but in only one case was the change less than 6·0 mg. per 100 cc. blood. The composite curve of this group shows none of the fluctuations commonly found both among normals and in Group 2.

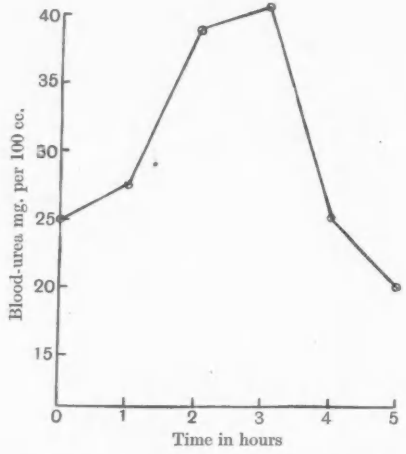

Fig. 2. Changes in the blood-urea of a case of melancholia after administration of 10 g. of glycine.

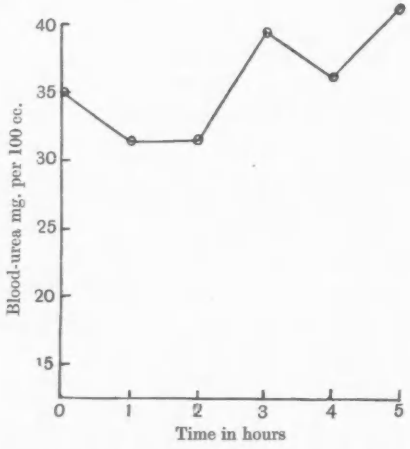

Fig. 3. Blood-urea changes in a case of hebephrenia after the administration of 10 g. glycine.

Group 2 is distinguished both from the normal and from Group 1 by the fact that at the 5th hour the value of the blood-urea is the highest observed during the test period, there being a relatively slow and gradual rise of the

urea level throughout the experimental period (*e.g.* Fig. 3). The successive changes up to the 5th hour were in no way regular and many cases yielded curves which exhibited a trough which varied in the time of its occurrence from individual to individual. The general extent of the final change is larger than normal but not so marked as in Group 1, and in many cases no differentiation based upon the actual changes could be made from the normal.

Melancholia.

Nineteen cases belonging to this group were investigated and it was found that all those in an acutely depressed or agitated condition belonged to Group 1 and gave the first type of curve (Fig. 2). The extent of the total changes appeared to be associated with the clinical state in that in the more agitated or depressed cases the urea level reached a higher peak and fell more rapidly than in the less severe cases. In three cases a high peak was reached as early as the 2nd hour and after a steady fall the urea showed a slight secondary rise in the last period. We repeated the test on one of these cases who had not changed clinically in the meantime after an interval of some weeks and found no change in the shape of the curve.

Six cases were found to give the second type of curve (Fig. 3) and were placed in Group 2; of these, three were showing good clinical improvement at the time of the test. They were in parole wards and interesting themselves in occupational therapy. Two others were retarded and listless and the last was in a good phase following continuous bath treatment.

A survey of the group as a whole leads to the conclusion that Fig. 2 is the characteristic finding in all agitated and acutely depressed cases while Fig. 3 occurs during improvement and in the less severe cases.

Relationship to emotional tension. The hyperglycaemic index, H.I. [McCowan and Quastel, 1931] is defined as "an expression of the sustainment of the hyperglycaemia found in many psychotic subjects" and its magnitude is a measure of the emotional tension of the patient, a finding corroborated by recent work on the galvanic skin reflex [Lockwood, 1932].

Taking the H.I. as the criterion we have found that the urea curve appears to offer no such objective standard of emotional tension; for although the H.I. was high in all the simple retarded cases giving the first type of curve, in two agitated cases presenting a curve of the same type it was zero. These findings suggest that Fig. 2 does not indicate the degree of emotional tension experienced by the subject.

Change of phase. In three cases, in whom no change in the physical or mental condition had been noted, the test was repeated after an interval of several weeks. The curve in each case presented no significant change. A fourth case was first investigated during a state of extreme agitation, when the curve obtained was of the first type showing a low fasting urea level with a marked and rapid rise and sharp fall to the fasting level. The test was repeated after

some months when he showed definite clinical improvement, the fasting urea level was now found to be normal and the curve approximated to that found in other cases showing improvement (Group 2).

Mania.

Two cases were investigated. They were examined in the acute stage within a week of admission and both were excited, restless, destructive and elated. In both these cases the fasting urea level was high and the curve showed little variation from the normal.

Table I.

				Mg. urea per 100 cc. blood Hours						
		Age	Sex	Initial	1	2	3	4	5	Remarks
Melancholia	1. R.L.	28	F.	24·75	26·7	31	39·6	35	30	Agitated
	2. C.W.	43	M.	10·2	24·4	27	31·4	19·8	11·2	,,
	3. R.G.	35	F.	13·5	10·1	15·4	19·8	14	12·8	,,
	4. N.L.	50	F.	31·5	32·5	41·5	39·7	—	36	,,
	5. A.L.	38	F.	30·5	31·5	30	37·5	—	33	,,
	6. L.T.	50	F.	14·5	24·6	35·7	27	24·3	26·5	,,
	7. A.G.	53	F.	31·5	—	43·5	36	30	34	,,
	8. L.B.	51	F.	31·5	31·5	29·5	34·5	39	37	,,
	9. P.C.	53	F.	21	22·5	25·5	34·5	—	25·5	,,
	10. J.E.	42	F.	33·9	31	39·6	31·8	33·4	36·4	Retarded
	11. B.A.	32	F.	24·8	27·8	39·3	41·4	25·5	21	,,
	12. A.C.	32	F.	27	30	34	40	—	27	,,
	13. B.J.	30	F.	27	30	28·5	34	—	30	,,
	14. A.J.	32	F.	27·5	21·3	—	25·5	30	34	After continuous baths
	15. B.D.	30	F.	18	16·5	19·5	18	—	37	Very retarded
	16. T.M.	49	M.	26·8	18·6	29	25·8	30	37	,,
	17. E.J.	22	F.	15·6	21·2	19·8	23·2	—	29·5	,,
	18. E.M.	—	F.	30	33	39	36	39	45	Improving
	19. G.S.	—	F.	21	24	33	36	—	36	,,
Mania	1. T.S.	43	M.	39	36	43	45	45	41	—
	2. H.G.	28	F.	40	41	43	43	45	41	—
Stupor	1. J.O.R.	38	M.	25·5	22·5	30	30	—	36	—
	2. G.G.	20	F.	25·5	22·5	24·5	24·5	—	84·9	—
Hebephrenia	1. D.L.	30	F.	27	30	30	33	—	33	—
	2. R.C.	32	F.	20	21·8	—	27	27	27	—
	3. G.W.	37	F.	35	31·8	31·8	39·4	36·8	42	—
	4. A.D.	28	F.	28	29	31·5	39	—	39	—
	5. R.N.	24	M.	25·5	19·5	21	21	21	31	—
	6. J.W.	33	M.	38	36·4	37	36·9	38	44·9	—
	7. H C.	27	M.	27	27·5	30	30	34·5	41·8	—
	8. W.I.	20	M.	21	22·5	25·5	28·5	25·5	31·5	—
	9. D.J.	35	F.	29·2	31·7	36·3	40	—	37·9	Excited
	10. T.L.	21	M.	8·55	15	9	19·2	22·8	10·05	,,
D. paranoides	11. J.B.	21	M.	42	36	36	41·5	42·5	45	—
	12. G.E.	33	M.	33	29·7	39	—	31·4	31·5	—
Catatonia	13. A.B.	21	M.	31	29·5	33	33	35·4	30	—
	14. G.N.	19	M.	23	26·5	27	30·6	27·8	28·5	—
	15. J.D.	38	M.	25·2	23·9	28·4	27	29	25·2	—
Mental defectives	1. N.R.	—	M.	28	21·5	31·5	28·5	35	36	—
	2. E.R.	—	F.	33	36	33	33	—	44	—

Stupor.

Two cases were investigated, one a catatonic stupor in a man aged 38 and the other a benign stupor occurring in a girl aged 20. In both the H.I. was zero. The results show both curves to be of the second type (Fig. 3), the initial changes fluctuating and of a small order and a sharp rise occurring at the 5th hour. Both these patients were fed on gelatin.

In the first of these cases the test was repeated after a month's interval; no change had been noted in the clinical state and the blood-urea curve showed no alteration.

Dementia praecox.

Fifteen cases were investigated, of whom eight emotionally flattened hebephrenics and one case of dementia paranoides gave curves of the second type (Fig. 3), while two hebephrenics, examined during a period of excitement, and one dementia paranoides conformed to the first type of curve (Fig. 2). In the remaining three cases, all catatonics, the curves approximated to the normal.

No definite conclusions can be drawn from these results but it appears that dull and apathetic states are associated with a relative delay in the rise of blood-urea.

In two mental defectives of unstable equilibrium the curves were of the second type.

Discussion.

A consideration of the above results (given in detail in Table I) shows that a disordered nitrogen metabolism is associated with abnormal mental states, the disorder being most marked in melancholia where the blood-urea curve is of a particular type showing rapid and uniform changes of a large order. The urea level rises to a sharp peak and falls rapidly. This type of curve (Fig. 2) occurs in all acutely depressed cases whether of the agitated or simple retarded type, and was not found in the only two cases of manic excitement investigated. It cannot depend exclusively upon the degree of emotional tension present since it occurred in two cases in which the H.I. was zero. From the cases investigated during improvement and from the few cases on whom we were able to repeat the test it appears that, as the clinical condition improves, the rate of change in the urea level becomes less marked so that the curve undergoes a certain amount of flattening, the maximum reading being observed in the 5th hour. The actual changes still remained fairly large. This second type of curve (Fig. 3) is found also in states of stupor and in the majority of apathetic hebephrenics. The changes found in the dementia praecox group on the whole, however, show marked variations between individuals and we have been unable to attach any significance to our findings in this group.

The evidence is insufficient to form any conclusions as to the chemical nature of the changes we have described but they appear to be due, either to an increased urea formation or to a disturbance of those factors which control the rate of entry into and disappearance from the blood of urea. In view of the fact that no disturbance of urea formation has been observed even in gross pathological states of the liver [Mann, 1927; Van Slyke and Stadie, 1920; Witts, 1929] the former possibility appears remote. The various facts confirmatory of the close connection between mental disorder and endocrine dysfunction, coupled with

the observations of Addis, Barnett and Shevky [1918], who showed that adrenaline and pituitrin regulate urea excretion, make the latter suggestion more feasible.

SUMMARY.

1. The blood-amino-acid changes following the administration of gelatin or glycine to 17 psychotic subjects of varying types have been observed. There was no material difference from the normal among the different clinical groups.

2. The blood-urea changes following glycine or gelatin ingestion have been noted in 41 cases of psychosis and in 5 normal controls.

3. The normal blood-urea curve shows variations from individual to individual but the total changes are of a small order the maximum value being observed within 5 hours of the ingestion of the amino-acid.

4. The characteristic blood-urea curve of agitated and simple retarded melancholia is typified by rapid and uniform changes, the total extent of the changes showing a marked increase over the normal. There is a rapid rise to a high level followed by a rapid fall within the 5-hour period to the fasting level.

5. A second curve is described in which, while the early changes are fluctuating, the maximum reading is obtained later than in the normal the actual changes again being larger than the normal. This is found to occur during recovery from melancholia and in apathetic states generally. The rise in urea level is slow, reaching its peak at the 5th hour of the test period.

6. The clinical and biochemical aspects of these changes are briefly discussed.

We wish to express our thanks to Dr P. K. McCowan, Medical Superintendent of the Cardiff City Mental Hospital, and Dr J. H. Quastel (Director of Research) for their help and criticism during the course of this investigation, and to the medical officers for access to their cases. One of us (D. R. D.) is indebted to the Medical Research Council for a whole-time assistance grant.

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XCI. THE DETERMINATION OF PYRUVIC ACID.

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In a previous paper [Case and Cook, 1931] reference was made to the need for an accurate quantitative method for the estimation of pyruvic acid in muscle and muscle-filtrates. The test used throughout the above work was the nitroprusside test of Simon and Piaux [1924]. This is sensitive and specific, the colour produced being easily distinguished from that given by any compound other than pyruvic acid. It is therefore extremely useful for the detection and approximate estimation of the latter. However, attempts which have since been made to develop a strictly quantitative colorimetric technique on these lines have proved fruitless owing to the fugacity of the colour and the fact that other substances present are liable to modify its tint.

It was thought that the phloroglucinol-HCl test of Posternak [1927] might be exploited, but although this is quite sensitive, the precise shade of colour is again interfered with by other substances to such an extent as to render it unsuitable for colorimetric purposes; moreover its specificity is not all that could be desired.

Methods based upon bisulphite-binding capacity [*e.g.* Cook, 1930] are not satisfactory, since pyruvic acid is far from being the only substance likely to occur in muscle which possesses this power. The same criticism applies to Wieland's [1924] method, depending upon iodoform formation.

Procedures involving reduction of the pyruvic acid to lactic acid and estimation of the latter compound [*e.g.* Lieben, 1923; Krishna and Sreenivasaya, 1928] are practically useless for muscle-filtrates on account of the large amounts of lactic acid already present.

Methods depending upon precipitation of pyruvic acid with phenylhydrazine and subsequent estimation of the unchanged hydrazine [MacLean, 1913] are clearly of no value when, as is the case in muscle, other hydrazone-forming substances are present also. Separation of the compounds formed with substituted hydrazines has hitherto been stated to be impracticable, since the solubility of the pyruvic acid derivatives is never sufficiently low to render possible anything approaching a quantitative precipitation. This obstacle has however been overcome in the present work, and a technique for the estimation

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of pyruvic acid in muscle-filtrates has been developed which depends upon the properties of the 2 : 4-dinitrophenylhydrazone of this substance.

Principle.

Ultimately this is similar to that of the method of Barrenscheen and Dreguss [1931] for the estimation of methylglyoxal; *viz.* the depth of colour produced by dissolving the 2 : 4-dinitrophenylhydrazone in alcoholic potassium hydroxide is determined colorimetrically against a standard. The difficulty raised by the appreciable solubility of this hydrazone in water is surmounted by extraction with ethyl acetate, followed by removal of the pyruvic acid derivative from the organic solvent by means of aqueous sodium carbonate. The corresponding hydrazones from a large number of other substances which may be present, *e.g.* methylglyoxal, acetaldehyde, *etc.* are not soluble in aqueous sodium carbonate.

Experimental details.

Deproteinisation of the tissue or tissue extract is carried out with trichloroacetic acid, which should be present in a final concentration of 2–5 %. 20 cc. of the clear filtrate are conveniently taken for the estimation, though if this quantity be not available, less can be used. (If more than 5 mg. of pyruvic acid are present in 20 cc. of filtrate it is advisable to dilute the latter accordingly and employ 20 cc. of the diluted solution.) 5 cc. of 1 % 2 : 4-dinitrophenylhydrazine (dissolved in 2 *N* HCl) are added and the mixture is kept for 2 hours at room temperature.

A visible precipitate may or may not appear, according to the amount of hydrazone-forming substances present. The whole is now shaken with 20 cc. of ethyl acetate in a glass-stoppered 50 cc. separating funnel. After separation, the aqueous layer, which is nearly colourless, is extracted with a further 10 cc. of ethyl acetate; as soon as the aqueous layer is colourless, as usually is the case after two extractions, it is discarded. The united ethyl acetate extracts now contain all the unchanged 2 : 4-dinitrophenylhydrazine together with the hydrazones which have been formed. The liquid is also acid, owing to the extraction of a certain amount of hydrochloric and trichloroacetic acids; these must be neutralised by shaking with solid calcium carbonate (Notes 2, 3). The solution is decanted into a glass evaporating basin, washing the calcium carbonate with further ethyl acetate until it is colourless. The washings are added to the main bulk of the fluid. The substances in solution must now be transferred to toluene (Note 4). This is accomplished by evaporating the contents of the dish on a water-bath to 1–2 cc. and then, after removal from the bath, adding about 20 cc. of toluene. The slightly cloudy yellow solution is again transferred to the separating funnel and is thoroughly shaken with 5 cc. of cold 25 % sodium carbonate solution (Note 5). If pyruvic acid was originally present its hydrazone dissolves in the aqueous layer, colouring it brown. This extraction is repeated with fresh Na_2CO_3 solution until the latter remains colourless; two or three repetitions usually suffice. The united sodium

carbonate layers are now acidified by adding concentrated hydrochloric acid drop by drop. The 2 : 4-dinitrophenylhydrazone of pyruvic acid is precipitated and a lemon-yellow suspension results. This is extracted in a separating funnel with successive 10 cc. portions of ethyl acetate until the aqueous layer is colourless (Note 6). It is not usually necessary to employ more than 20 cc. of ethyl acetate to achieve this. The ethyl acetate solution now contains all the pyruvic acid hydrazone which was present, and is evaporated to dryness in a glass basin on a water-bath. The yellow residue is dissolved when cool in 5 % alcoholic potassium hydroxide (Note 7), giving a deep red solution which is made up in a graduated vessel with further alcoholic potash to a volume such that the colour is suitable for reading in a colorimeter. In practice it is found that the total volume at this stage should be about 50 cc. for every mg. of pyruvic acid that was originally present in the sample taken.

For the purpose of the standard, a pure preparation of pyruvic acid 2 : 4-dinitrophenylhydrazone is made, and a stock solution of this is kept in ethyl acetate, of such a strength that 1 cc. is equivalent to 0·1 mg. pyruvic acid. The appropriate volume is taken, evaporated to dryness, and dissolved in alcoholic potash exactly as described above. As in all colorimetric work, the standards should be chosen so that the depth of colour obtained is as close as possible to that in the unknown when both are made up to the same volume. Preliminary tests show, however, that in the neighbourhood of the concentrations stated proportionality exists between the depth of colour and the amounts of hydrazone present.

Notes. 1. If sulphite or bisulphite is present, the acid filtrate should be boiled until no more SO_2 is given off. Sulphite interferes with the precipitation to some extent.

2. If this neutralisation be omitted, the subsequent heating of the solution leads to the formation of small quantities of compounds other than the pyruvic acid derivative which are extracted by the sodium carbonate, colouring it brown, and which are afterwards precipitated upon acidification, giving a reddish colour in the final treatment with alcoholic potash. When the neutralisation is properly carried out, the sodium carbonate always remains colourless in blank determinations, *i.e.* when no pyruvic acid is present.

3. It was at first the practice to neutralise the acid solution before extraction with ethyl acetate, but if this be done it is found that extraction has to be repeated a much larger number of times before the aqueous layer is colourless.

4. The solubility of the pyruvic acid derivative in ethyl acetate is so great that it is not completely removed by sodium carbonate solution. Moreover, shaking the ethyl acetate solution with sodium carbonate seems to lead to a small amount of saponification, with the result that separation into layers is imperfect. The substitution of toluene for ethyl acetate overcomes both of these difficulties, but it cannot be used for extraction at the outset because the hydrazine and hydrazones are not sufficiently soluble in it to render the process efficient.

5. The toluene extract and the sodium carbonate solution must both be at room temperature. Warm Na_2CO_3 reacts with other 2 : 4-dinitrophenylhydrazones besides that of pyruvic acid.

6. A good deal of care must be taken at this stage that the effervescence produced by the liberated CO_2 does not lead to loss by splashing.

7. A small quantity of sodium chloride and traces of trichloroacetate are always present at this stage, having been extracted by the ethyl acetate. It is advisable therefore first to add a drop or two of water to dissolve these substances, which may otherwise cause the final solution to be turbid. By reason of the presence of these admixtures, actual weighing of the hydrazone is impracticable.

Before giving experimental figures obtained by this method, it is worth while pointing out that pyruvic acid very readily undergoes changes of the nature of polymerisation or condensation, and that the purity of laboratory samples is therefore always of a low order. For the purposes of this work, Kahlbaum's pyruvic acid was twice distilled *in vacuo*, the fraction boiling at about 66°/15 mm. being collected. The colourless product was diluted with ice-cold water and accurately neutralised with sodium hydroxide, the temperature being kept below 5°. The solution was now concentrated in a vacuum desiccator; the glistening white plates of sodium pyruvate that crystallised out were powdered, washed thoroughly with boiling absolute alcohol, heated for some time at 100°, and stored *in vacuo* over sulphuric acid. In the determinations reported below, the pyruvic acid was added in the form of equivalent amounts of this sodium salt in aqueous solution of known concentration.

Table I sets forth the results of determinations made by this method when quantities of pyruvic acid varying between 0·1 mg. and 10·0 mg. were taken. It also shows that the presence of trichloroacetic acid does not interfere.

Table I.

Pyruvic acid dissolved in	Pyruvic acid taken (mg.)	Pyruvic acid found (mg.)	Percentage error
20 cc. water	0·10	0·094	−6·0
,,	0·15	0·145	−3·3
,,	0·20	0·200	0·0
,,	0·50	0·498	−0·4
,,	1·00	1·018	+1·8
,,	2·00	1·980	−1·0
,,	5·00	4·900	−2·0
,,	10·00	9·130	−8·7
20 cc. 5 % trichloroacetic acid	0·10	0·096	−4·0
,, ,,	0·15	0·147	−2·0
,, ,,	0·20	0·204	+2·0
,, ,,	0·50	0·490	−2·0
,, ,,	1·00	0·980	−2·0
,, ,,	2·00	2·000	0·0
,, ,,	5·00	4·880	−2·4
,, ,,	10·00	9·300	−7·0

It is to be seen from this table that under the conditions described the method works most satisfactorily for quantities of pyruvic acid between 0·2 mg. and 5·0 mg., which are estimable with a possible error not exceeding ± 2 %. Since a concentration of 0·2 mg. in 20 cc. represents a dilution of 1 part in 100,000, considerable sensitivity as well as accuracy can be claimed.

In Table II it is shown that the estimation is not interfered with by the presence of comparatively large amounts of a variety of other metabolites which may possibly be present in preparations from muscle. The pyruvic acid and other substances are dissolved throughout in 20 cc. of 5 % trichloroacetic acid.

The method has thus a high degree of specificity. Most of the substances in Table II form hydrazones with 2 : 4-dinitrophenylhydrazine, many of which give a red colour with alcoholic potash; but, of them all, pyruvic acid is

Table II.

Pyruvic acid taken (mg.)	Additions other than pyruvic acid	Pyruvic acid found (mg.)	Percentage error
0·20	50 mg. glucose	0·197	−1·5
5·00	,, ,,	5·050	+1·0
0·20	50 mg. fructose	0·194	−3·0
5·00	,, ,,	4·890	−2·2
0·20	50 mg. glycogen	0·200	0·0
5·00	,, ,,	5·044	+0·9
0·20	50 mg. starch	0·198	−1·0
5·00	,, ,,	4·900	−2·0
0·20	50 mg. methylglyoxal	0·202	+1·0
5·00	,, ,,	5·080	+1·6
0·20	50 mg. dihydroxyacetone	0·196	−2·0
5·00	,, ,,	5·065	+1·3
0·20	50 mg. glyceraldehyde	0·200	0·0
5·00	,, ,,	4·920	−1·6
0·20	50 mg. sodium hexosediphosphate	0·198	−1·0
5·00	,, ,,	5·050	+1·0
0·20	50 mg. sodium hexosemonophosphate	0·205	+2·5
5·00	,, ,,	5·092	+1·8
0·20	50 mg. acetaldehyde	0·200	0·0
5·00	,, ,,	4·950	−1·0
0·20	50 mg. formaldehyde	0·194	−3·0
5·00	,, ,,	4·900	−2·0
0·20	50 mg. acetone	0·203	+1·5
5·00	,, ,,	5·000	0·0

the only one whose hydrazone is extracted by sodium carbonate. It is probable that this property is associated with the presence in the molecule of the —COOH group, in addition to the ketonic group which is responsible for the formation of the hydrazone. For this reason other compounds containing groups of both these types would be expected to interfere. To take two such examples, glycuronic acid and laevulic acid have been tested. Both of these substances form hydrazones with 2 : 4-dinitrophenylhydrazine which are yellow, and which are soluble in aqueous sodium carbonate; both hydrazones yield a red colour with alcoholic potash. By this method, therefore, such compounds would be estimated as pyruvic acid. But in working with muscle, at all events, it is practically certain that the results obtained are to be ascribed in reality to pyruvic acid, and that no interfering substances of the type just discussed are present, for the following reasons.

(*a*) The results given by the method described invariably run closely parallel with observations made by means of the nitroprusside test; this is exemplified in another communication [Case, 1932].

(*b*) If the product obtained in the final stages of the procedure be further purified and examined, no hydrazone can be isolated from it other than one melting at 214°, and thus corresponding to pyruvic acid [Allen, 1930].

SUMMARY.

A method is described for the determination of pyruvic acid, with special application to muscle-filtrates. One part in 100,000 of pyruvic acid can be estimated, in absolute amounts at least as low as 0·2 mg. The specificity is discussed and shown to be of a high order.

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XCII. THE ORIGIN OF PYRUVIC ACID IN MUSCLE.

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Introduction.

It was shown by Case and Cook [1931] that it is possible under a variety of conditions to detect the presence of methylglyoxal and pyruvic acid among the products of the metabolism of muscle. With regard to methylglyoxal in particular, our results constituted an addition to the already considerable quantity of evidence which has accumulated in favour of the hypothesis that this substance is of importance in intermediary carbohydrate metabolism. From purely chemical considerations, the participation of methylglyoxal in the transformation of carbohydrate to lactic acid has long appeared probable; biological evidence for such a view may be said to have begun with Dakin and Dudley's [1913] demonstration of the existence in animal tissues of the enzyme glyoxalase, which converts α-ketonic aldehydes into the corresponding hydroxy-acids. At about the same time Neuberg [1913] observed the conversion of methylglyoxal to lactic acid in tissues, and Levene and Meyer [1913] showed that leucocytes could bring about the same transformation. Since then evidence has accumulated which has established conclusively the facts that diverse animal tissues which are known to produce lactic acid are also capable of forming methylglyoxal, and that methylglyoxal is transformed to lactic acid by these tissues as readily as, or more readily than, carbohydrates. These two circumstances, taken in conjunction with the widespread occurrence of glyoxalase in nearly all tissues, make the probability strong that methylglyoxal is indeed a normal precursor of lactic acid. Some stress is laid upon this because, in what follows, this hypothesis is assumed.

In addition to methylglyoxal, Case and Cook were able to identify the corresponding keto-acid, pyruvic acid, as a metabolite under similar conditions. They were led to make the tentative suggestion that this pyruvic acid which appeared was a product, not of lactic acid oxidation, but of the chain of processes leading up to the formation of lactic acid.

Such a conclusion is supported by the results of Utewski [1929], who estimated the lactic acid produced by autolysing muscle in the presence and absence of sodium pyruvate, and reported that much more lactic acid appeared when pyruvate was added. Mendel, Bauch and Strelitz [1931] found that the

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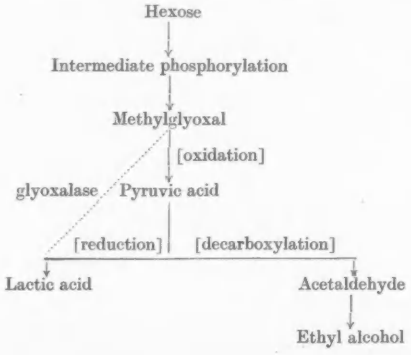
anaerobic glycolysis of animal tissues is more than doubled by the addition of pyruvic acid, and they also state that tumour cells, which are remarkable for their high glycolytic activity, produce pyruvic acid in large amounts. Hahn and Haarmann [1930] found that muscle which had been thoroughly washed, and hence presumably freed from lactic dehydrogenase (as Hahn and Fischbach [1929] had previously stated), readily produced pyruvic acid from fructosediphosphoric acid.

That in tissues other than muscle, pyruvic acid can be reduced to lactic acid, appears likely from the work of Khouvine, Aubel and Chevillard [1929, 1930] who in a series of investigations demonstrated the power of liver to bring about this transformation either aerobically or anaerobically. It has been shown [*e.g.* Mayer, 1912] that lactic acid can be found in the urine of animals which have been given pyruvic acid by mouth. It is of course not permissible to draw any conclusions with regard to the metabolism of muscle from observations on the liver, and the above examples are mentioned simply as indicating that the change pyruvic acid → lactic acid can and does occur in some animal tissues. Again, it is unsafe to draw too close an analogy between lactic fermentation by muscle and fermentations by yeast and bacteria. It is important to remember, however, that it has been thoroughly established by Neuberg and his associates that the formation of pyruvic acid is a normal stage in the fermentation of carbohydrate by yeast; but ordinarily it undergoes subsequent changes which do not occur in muscle. As an indication of the part played by pyruvic acid in lactic fermentation by bacteria, we may cite its isolation by Kostychev, Gwaladse and Eliasberg [1930], who employed semicarbazide as a fixative in the lactic fermentation by *B. caucasicum*.

It has recently been made fairly certain [*e.g.* by Neuberg and Kobel, 1928, 1929, 2; by Gottschalk, 1928; and by Bayo, 1929], by the use of co-enzyme-free yeast preparations, that the formation of methylglyoxal is the step intermediate between phosphorylation and the formation of pyruvic acid. The same has been shown to be true with respect to certain lactic acid-forming organisms, as for instance by Neuberg and Kobel [1929, 1] in the case of *B. delbrücki*.



These changes may be expressed as above (following the continuous lines). The left-hand branch represents the case of lactic acid-forming organisms, or yeast under some special conditions, while the right-hand branch represents ordinary alcoholic fermentation.

But animal tissues are strongly characterised by their possession in marked degree of the enzyme glyoxalase, which converts methylglyoxal directly into lactic acid; this transformation appears to be effected by an addition of the elements of water so that the change methylglyoxal → lactic acid takes place in one step. In other words, an internal oxidation-reduction seems to be involved, in contradistinction to a phase of oxidation (with formation of pyruvic acid) followed by one of reduction (with formation of lactic acid). The scheme given above must therefore be amplified by addition of the direct conversion of methylglyoxal into pyruvic acid, represented by the dotted line, to include the case of animal tissues [*cf.* Gottschalk, 1926].

By such considerations as these a good deal of doubt is cast upon the likelihood of pyruvic acid formation as a stage in the course of normal lactic acid formation in muscle, and shortly after the completion of the above-mentioned paper by Case and Cook the present author began to feel that the validity of such a hypothesis was questionable. The purpose of this communication is to report some of the experimental work which has been performed since that time and to discuss its bearing upon the problem of pyruvic acid formation and oxidation in general in muscle.

Experimental.

Methods.

Pyruvic acid was used in the form of its sodium salt, prepared in the manner described in a preceding paper [Case, 1932]. The method used for its determination is given in full in the same place. Continued use has also been made of the Simon and Piaux [1924] reaction which was employed in former work.

Solutions of methylglyoxal were obtained either by the method of Neuberg and Hofmann [1930] or by the following procedure: *iso*nitrosoacetone (methylglyoxal aldoxime) was prepared from acetone and the *iso*nitroso-group removed by boiling with dilute sulphuric acid [Pechmann, 1887]. On distilling, the methyglyoxal was contained in the distillate. This latter method was found on the whole the more satisfactory, as it is extremely difficult by Neuberg and Hofmann's procedure to obtain a product free from impurity, notably formaldehyde. The solutions of methylglyoxal could be further purified by redistillation with phosphorus pentoxide and concentration *in vacuo*. The methylglyoxal was standardised in the first place by weighing the yield of 2 : 4-dinitrophenylosazone obtained from a known volume, and subsequent estimations were carried out by a slight modification of the method of Barrenscheen and Dreguss [1931] which, although laborious, seems to be the most satisfactory yet devised.

The preparation of muscle extracts and the determinations of lactic acid were performed exactly as has been customary in former work.

The first experiments to be reported are concerned with the confirmation of the previous findings that while pyruvic acid production is increased by the addition of starch or glycogen, the addition of lactate has little effect. Flasks were set up with the following contents, and incubated at room temperature:

	A (cc.)	B (cc.)	C (cc.)
Rabbit muscle extract	50	50	50
Phosphate buffer, p_H 7·2 (10 times strength)	5	5	5
M Na_2SO_3	3	3	3
2 % soluble starch	—	10	—
$M/5$ sodium lactate	—	—	10
Water	to 100 cc.		

Nitroprusside reactions were carried out from time to time upon 3 cc. samples, and at the end of 3 hours pyruvic acid estimations were performed. The results appear in Table I.

Table I. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B	C
0	–	–	–
20	–	+ +	–
60	+	+ + +	+ –
120	+	+ + + + +	+
180	+	+ + + + +	+

Pyruvic acid after 180 mins., expressed as mg./100 cc. incubation mixture.

A	B	C
7	53	8

It is obvious that while the presence of starch has given rise to a large increase of pyruvic acid over the amount formed in the control, the addition of lactate has had no such effect. It is to be noted however that the experiment was carried out with muscle extract, in common with most of the rest of the work to be reported in this paper. Since there is no recorded instance of a lactic dehydrogenase having been obtained in an aqueous extract from muscle, it cannot be concluded from the above result that in muscle tissue itself pyruvic acid does not originate from lactate. Therefore the experiment was repeated using chopped rabbit muscle. Also, since it was thought possible that the configurative difference between the *dl*-lactate added and the lactic acid actually produced by muscle itself might account for the result, flasks were included in which a preparation was added which had been made by mincing rabbit muscle, allowing to stand for 2 hours, extracting with buffer, boiling, filtering and concentrating *in vacuo*. This preparation was found to contain about 30 mg. of lactic acid per 10 cc.

The experimental flasks were as follows:

	A	B	C	D
Chopped rabbit muscle	10 g.	10 g.	10 g.	10 g.
Buffer, p_H 7·2 (× 10)	3 cc.	3 cc.	3 cc.	3 cc.
M Na_2SO_3...	2 cc.	2 cc.	2 cc.	2 cc.
2 % soluble starch	—	5 cc.	—	—
M/5 sodium lactate	—	—	5 cc.	—
Muscle-lactic acid preparation ...	—	—	—	30 cc.
Water	to 50 cc.			

The results of the periodical nitroprusside tests, and the final estimations of pyruvic acid, are given in Table II.

Table II. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B	C	D
0	–	–	–	+
60	–	+ + +	–	+
120	–	+ + + +	+ –	+

Pyruvic acid after 120 mins., expressed as mg./100 cc. incubation mixture.

A	B	C	D
4·5	69	9·5	18

Here again then the findings are similar. The flask containing starch shows a great increment in pyruvic acid content. There is a slight increase in those containing lactate, the possible significance of which will be discussed later on. The slight positive reaction shown throughout by the contents of flask D is clearly due to preformed pyruvic acid in the added preparation.

Similar experiments, employing chopped frog muscle instead of rabbit muscle, gave results of the same type. Weight for weight, rather more pyruvic acid was formed by the frog muscle than by the rabbit muscle.

The next subject for investigation was the effect upon pyruvic acid production of agents which inhibit the formation of lactic acid from carbohydrates. Sodium fluoride, which prevents the breakdown of phosphoric esters, and amylase, which inhibits at an earlier stage [Case and McCullagh, 1928], were of course obvious substances to try. A typical experiment is recorded below:

	A (cc.)	B (cc.)	C (cc.)
Muscle extract ...	50	50	50
Buffer, p_H 7·2 (× 10) ...	5	5	5
M Na_2SO_3	3	3	3
2 % soluble starch ...	10	10	10
M/10 sodium fluoride...	—	10	—
Saliva (1 in 5) ...	—	—	10
Water	to 100 cc.		

(It was known from experience that these quantities of sodium fluoride and dilute saliva were sufficient to cause complete suppression of lactic acid formation.)

Incubation was, as usual, at room temperature; nitroprusside tests were performed initially and at intervals. Table III gives the results.

Table III. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B	C
0	–	–	–
20	+ + +	–	–
60	+ + +	–	–
90	+ + + + +	–	–

Pyruvic acid after 90 mins., expressed as mg./100 cc. incubation mixture.

A	B	C
65	3	4·5

This experiment then shows that both amylase and sodium fluoride, which are powerful inhibitors of glycolysis, have completely suppressed the formation of pyruvic acid which otherwise takes place to a large extent from starch. Some experiments which were done with iodoacetic acid will be reported in detail in a later communication, as their significance is not fully clear. So far, it has been found that concentrations of this substance which totally inhibit lactic acid production may bring about only a partial inhibition of pyruvic acid formation. In this connection it has to be remembered (*a*) that iodoacetic acid may exert its inhibitory action at two stages of glycolysis at least [Dudley, 1931], (*b*) that in higher concentrations it inhibits respiration as well as glycolysis, and (*c*) that its effect is not instantaneous. Hence in drawing conclusions from experiments involving its use there are a number of factors to be taken into account.

Now, so far as all these results go, they certainly indicate that for pyruvic acid to be formed, glycolysis must occur and must not be inhibited. But they do not settle the problem as to whether pyruvic acid is actually a stage in lactic acid formation, though such a hypothesis is not contradicted by the facts so far reported. More light can be thrown upon this question by experiments to determine the fate of pyruvic acid when it is added to muscle. Some of the evidence in the literature which suggests the possibility that pyruvic acid goes to lactic acid has already received mention in the introduction.

It will be remembered that in the work of Case and Cook [1931] added pyruvic acid was found to disappear rapidly from minced muscle. Before the publication of that paper, a rough experiment had been done which indicated that a corresponding increase of lactic acid took place, as had been found by Utewski [1929], and it was intended to repeat this later on, making accurate determinations. When the present author attempted to do this, however, it was found to be a less straightforward undertaking than had been supposed. The results were extremely variable in that sometimes pyruvic acid disappeared at a rapid rate and sometimes at a slow one. In three cases only, out of sixteen tried, was there any formation of lactic acid in excess of the amount simultaneously formed in the controls without added pyruvate, and even then the increase accounted for less than 10 % of the pyruvate disappearing. This series of sixteen tests embraced experiments with minced

rabbit muscle, rabbit muscle extract, and chopped frog muscle, at room temperature and at 37°. It is to be noted here that Toenniessen and Brinkmann [1930] and Toenniessen [1930] showed that if pyruvic acid were perfused through the musculature of eviscerated rabbits it very rapidly disappeared. But it did not form lactic acid; succinic acid and formic acid made their appearance.

There cannot be said then to be sufficient evidence for the belief that the transformation of carbohydrate to lactic acid in muscle involves the intermediate formation of pyruvic acid, and some other explanation must be sought for the appearance of this latter substance. It is at least certain from the work already reported that pyruvic acid occurs in the greatest quantities under conditions which are accompanied by breakdown of carbohydrate to lactic acid, *viz.* glycolysis *in vitro*, and activity as a result of stimulation *in vivo*. It might be argued from this that the result of glycolysis is merely to give rise to the production of large amounts of lactic acid, from which pyruvic acid is formed by a simple dehydrogenation. But this is rendered unlikely by the fact, demonstrated by Case and Cook and here confirmed, that the formation of pyruvic acid brought about by the addition of lactate to the system is not comparable in amount with that brought about by the addition of carbohydrate. If also, then, pyruvic acid is to be excluded as an intermediary in glycolysis, the most feasible supposition remaining is that it is formed as a result of a side-reaction from something which is itself an intermediate. Methylglyoxal of course suggests itself. The next experiments to be described deal therefore with the influence of added methylglyoxal upon the appearance of pyruvic acid.

The following incubations were made:

	A	B
Muscle extract ...	50 cc.	50 cc.
Buffer, p_H 7·2 (× 10)...	5 cc.	5 cc.
M Na_2SO_3	3 cc.	3 cc.
Methylglyoxal ...	0·2 g.	—
Water	to 100 cc.	

Table IV. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B
0	–	–
20	–	–
60	+ –	–
120	+	+
240	+ +	+
18 hours	+ + +	+

Pyruvic acid after 4 hours, expressed as mg./100 cc. incubation mixture.

A	B
15	6

Pyruvic acid after 18 hours.

A	B
39	9·5

After 4 hours, it is thus seen, there was surprisingly little formation of pyruvic acid from methylglyoxal, compared with the amounts obtained from an equivalent quantity of carbohydrate. It is true that after standing overnight a considerable increase in pyruvic acid was found to have occurred, and this is recorded in Table IV; but of course a figure obtained after so long an incubation is of doubtful value owing to the possibility of bacterial contamination. It was at first thought that the experiment might be faulty, but after a large number of repetitions the above results were shown to be quite typical.

As a necessary control, an experiment without sulphite was performed, lactic acid being estimated instead of pyruvic acid, and it was found that about 40 % of the methylglyoxal was transformed rapidly into lactic acid. The details of this are as follows:

	A	B
Muscle extract ...	50 cc.	50 cc.
Buffer, p_H 7·2 (× 10)...	5 cc.	5 cc.
Methylglyoxal ...	—	0·2 g.
Water	to 100 cc.	

Table V.

Methylglyoxal, expressed as mg./100 cc. incubation mixture.

Time after commencement of exp. (mins.)	A	B
0	8	203
15	6	38
60	6	8

Lactic acid, expressed as mg./100 cc. incubation mixture.

Time after commencement of exp. (mins.)	A	B
0	47	51
15	58	125
60	63	136

In this control, without sulphite, the nitroprusside tests were throughout negative, or at most faintly positive. The fate of that portion of the methylglyoxal which under these conditions disappears but does not give rise to lactic acid is not at present clear; the same phenomenon has been encountered by many other workers.

Hexosediphosphate was next tried as a substrate, and was found in presence of sulphite to yield pyruvic acid to about the same extent as did starch, and therefore much more readily than did methylglyoxal. An experiment is reported below. The sodium hexosediphosphate which was used here was prepared from the calcium salt in solution by quantitative decomposition with sodium oxalate.

	A	B	C	D
Muscle extract ...	50 cc.	50 cc.	50 cc.	50 cc.
Buffer, p_H 7·2 (× 10) ...	5 cc.	5 cc.	5 cc.	5 cc.
M Na_2SO_3	3 cc.	3 cc.	3 cc.	3 cc.
Na hexosediphosphate...	—	—	0·4 g.	0·4 g.
Methylglyoxal	—	0·2 g.	—	0·2 g.
Water	to 100 cc.			

The results are set forth in Table VI.

Table VI. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B	C	D
0	–	–	–	–
20	–	–	++	++
60	+–	–	+++	+++
120	+	+	+++++	+++++
18 hours	++	+++	+++++	+++++

Pyruvic acid after 2 hours, expressed as mg./100 cc. incubation mixture.

A	B	C	D
8	12	71	77

Pyruvic acid after 18 hours.

A	B	C	D
14·5	49	88	113

It is clear from this that pyruvic acid is formed very much more rapidly from hexosediphosphate than from methylglyoxal. Once again, after standing overnight, there is very appreciable production of pyruvic acid in the flask with added methylglyoxal. Although this probably indicates a slow reaction of the same type as the comparatively rapid one occurring in flask C, it is subject to the criticism already made in connection with the corresponding results in Table IV. Flask D is a control intended to demonstrate that the slow formation of pyruvic acid from methylglyoxal as compared with that from hexosediphosphate is not due to poisoning of the enzyme system by impurities present in the methylglyoxal preparations used, as seemed quite possible. It is seen that addition of methylglyoxal does not retard the production of pyruvic acid from hexosediphosphate.

The last set of experiments to be described in the present communication is concerned with the effect of antiglyoxalase on the formation of pyruvic acid. The following flasks were set up:

	A	B	C	D	E	F
Muscle extract	50 cc.	50 cc.	50 cc.	50 cc.	50 cc.	50 cc.
Buffer, p_H 7·2 (× 10) ...	5 cc.	5 cc.	5 cc.	5 cc.	5 cc.	5 cc.
M Na_2SO_3	3 cc.	3 cc.	3 cc.	3 cc.	3 cc.	3 cc.
Soluble starch	0·2 g.	0·2 g.	—	—	—	—
Na hexosediphosphate...	—	—	0·4 g.	0·4 g.	—	—
Methylglyoxal	—	—	—	—	0·2 g.	0·2 g.
Antiglyoxalase	—	10 cc.	—	10 cc.	—	10 cc.
Water	to 100 cc.					

The antiglyoxalase here used was made from an aqueous extract of commercial pancreatin which had been digested for 24 hours with 1 % sodium carbonate to remove trypsin and amylase. Tests showed that the preparation was active as an antiglyoxalase. The muscle extract used in the above experiments was incubated for 2 hours at 37° with the antiglyoxalase and buffer before adding the other substances, while in those flasks from which antiglyoxalase was to be absent, the muscle extract and buffer alone were

incubated at 37° for the same length of time. The subsequent "experimental" incubation was, as usual, at room temperature.

Table VII. *Nitroprusside reactions.*

Time after commencement of exp. (mins.)	A	B	C	D	E	F
0	–	–	–	–	–	–
60	++	+++	++	+++	–	+–
120	+++	++++	++++	+++++	+–	++
18 hours					++	++++

Pyruvic acid, expressed as mg./100 cc. incubation mixture.

	A	B	C	D	E	F
After 2 hours	47	88·5	59	104	10	15·5
After 18 hours					33	48

In all three cases it is to be observed that the presence of antiglyoxalase has brought about an increase in the quantity of pyruvic acid which appears. As in the preceding experiments, the production of pyruvic acid from methylglyoxal is very much slower and less marked than from starch or hexose-diphosphate.

Discussion.

The question of the origin of pyruvic acid in muscle assumes great significance if the hypothesis be regarded as legitimate that the occurrence of this substance is associated with the oxidative phase of the much-discussed "carbohydrate cycle." A clue may be afforded by the present work as to the actual stage of the cycle where oxidation comes into play. In considering the aerobic removal of lactic acid it has long been a disputed problem as to whether part of the lactic acid itself is the material which is oxidised in order to provide energy for the resynthesis of the remainder, or whether some other member of the metabolic chain is more directly concerned. On the assumption that lactic acid as such undergoes oxidation, the operation of a lactic dehydrogenase is generally postulated, which would yield pyruvic acid as the first oxidation product. And indeed the phenomena which are observed if lactic acid be added to an intact muscle seem to be entirely compatible with this conception; oxidation does take place, and pyruvic acid is formed and can be detected by suitable means. Nevertheless, it has not so far proved possible to obtain from muscle an extract possessing the property of a lactic dehydrogenase, and even if the tissue be merely chopped or minced so that its structure is disintegrated it now yields pyruvic acid very much less readily from added lactate, as is shown in the present work. It is well known that the synthetic activity of muscle is severely impaired by such treatment as chopping, and may be destroyed altogether. It appears quite likely that this is connected with the simultaneous loss of the power of producing pyruvic acid from lactic acid; in other words, the formation of pyruvic acid from lactic acid may result not from direct oxidation of the latter, but from the oxidation of some compound intermediary between lactic acid and carbohydrate. On the whole there has,

until recently, been little available evidence to assist in arriving at a decision in favour of one or the other of these alternatives. The information afforded by the present work points rather strongly to the probability that the second is the correct one.

Results obtained by methods involving the use of tissue extracts must always be interpreted with circumspection when they are applied to considerations of events taking place in intact tissues or organs; at the same time, the employment of such procedures has a definite advantage in that it facilitates a certain degree of separation and differentiation between various correlated systems and processes. In the present instance, for example, the use of muscle extracts eliminates complications ensuing from what may be called the synthetic half of the carbohydrate cycle. The fact that in such a system pyruvic acid formation can still be observed, while lactic acid when once formed plays no further part in the metabolic changes, may be of prime importance from the point of view of the problem under discussion.

Again, the fact must be borne in mind that throughout this work a fixative, namely sodium sulphite, has been employed. This of course is quite justifiable when, as was here the case, the primary object was to gain information as to the precursor of pyruvic acid. The actual magnitude of the extent of the reaction thus brought into prominence may or may not be considerably modified by the use of such a technique, and this has to be remembered when attempting to relate the findings thereby obtained with the extent to which the process occurs under normal conditions. The modification in this case can only be a quantitative, and not a qualitative one, however, for it has previously been shown that it is possible to demonstrate the presence of pyruvic acid in muscle in the absence of any fixative.

When we come to consider the identity of the precursor of pyruvic acid, some interesting points are raised. The experiments with inhibitors show that this precursor must occupy a place in the glycolytic chain subsequent to that occupied by hexosephosphoric esters. It has been shown that it is extremely unlikely to be lactic acid. Therefore, the only substance definitely known to occur which fulfils the necessary conditions is methylglyoxal (see the scheme given in the introduction). It seems very probable then that pyruvic acid arises from methylglyoxal, and by analogy with the generally accepted theory of yeast fermentation this probability is strengthened. This suggestion has been made before, *e.g.* by Toenniessen and Brinkmann [1930], and the present work certainly lends it support. It seems at first sight surprising therefore that, as has been shown here, added methylglyoxal does not readily yield pyruvic acid, while hexosediphosphate does. But consideration shows that a parallel exists in the case of yeast fermentations where, although it is practically sure that methylglyoxal is an intermediate, the fact remains that added methylglyoxal is almost unfermentable. This is explained by the supposition that the methylglyoxal participates in the fermentation in a form which is not structurally identical with that of extraneously added methylglyoxal. Precisely

in what this difference consists is still a matter for conjecture. It seems very possible that methylglyoxal as actually produced in fermentation either by muscle or by yeast is in the form of a hydrate, in which case the formation of pyruvic acid would be the result of a direct dehydrogenation, while the action of glyoxalase would be a structural rearrangement, the necessary elements of water being already present in the molecule. This hypothesis, though not a new one by any means, is very attractive and would account for the results reported in this paper. On the other hand, it has been suggested [*e.g.* Giršavičius, 1930], with some experimental support, that the $CH_3.CO.CHO$ form is the fermentable one, while the hydrated form is the one existing in solutions of the substance. Again, other suggestions postulating the existence of tautomeric forms have been put forward, notably by Neuberg. At present it is not possible to decide which is the correct interpretation of the known facts, but the data presented in this paper almost certainly call for explanation on these lines. The experiments with antiglyoxalase carry a good deal of significance, since they indicate that when the methylglyoxal is prevented from being transformed to lactic acid—the reaction which normally preponderates—it is more available for the process which results in the appearance of pyruvic acid. Many interesting issues are involved which must be made the subject of future work. For example, in view of what has been said above the possibility arises that there may in reality be no true lactic dehydrogenase in muscle. This question is at present receiving attention.

SUMMARY.

The occurrence of pyruvic acid in muscle is more fully investigated with the object of ascertaining its mode of origin. The conclusion is drawn that it is not an intermediate in lactic acid formation and that it is not a product of direct oxidation of lactic acid. It is suggested that it is derived from the modification of methylglyoxal which is widely believed to participate in carbohydrate metabolism. The problem is discussed in relation to oxidative processes in muscle and the situation with regard to methylglyoxal in fermentations in general.

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XCIII. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

III. THE RELATION OF AMINO-ACID ABSORPTION TO PROTEIN SYNTHESIS.

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The work of Crowther and Raistrick [1916] indicates that the globulin of milk is identical with the globulin of blood. Lactalbumin and caseinogen appear, however, to be specific products of mammary secretion. Cary [1920], using the technique of Kaufmann and Magne [1906], demonstrated that the amino-acid content of blood obtained from the mammary vein of lactating cows was significantly lower than that obtained from the jugular vein. From this fact he concluded that the amino-acids of the blood are the precursors of the milk-proteins.

In view of the work of Delaunay [1913], Folin and Denis [1912], and Van Slyke and Meyer [1912] on the rôle of amino-acids in protein metabolism, such an assumption is *a priori* a reasonable one. Cary's conclusions are, however, open to criticism since he arbitrarily assumed that the amino-acid content of jugular blood could be taken as representative of the arterial blood flowing to the mammary gland. In the first paper of this series [Blackwood and Stirling, 1932, 2] it has been shown that the intervention of salivary secretion in the path of blood traversing the tissues of the head and neck necessitates a correction in the analytical values for jugular blood. In addition, no comparative values for the amino-acid contents of jugular and arterial bloods are to be found in the literature, so that Cary's assumption that these are identical appears unwarranted.

Apart from these criticisms, the method of blood analysis used by Cary was long and tedious, and involved so many separate processes (including incubation with urease, deproteinisation with boiling dilute acetic acid, treatment with kaolin, and subsequent concentration *in vacuo*) that considerable errors might be anticipated.

In view of these facts it appeared desirable to re-investigate the whole problem, using methods which were not open to the above criticisms. The methods adopted in the present investigation differ in three essentials from

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those of Cary. Estimations have been made of iron content of the blood samples, so that correction might be allowed for the water absorbed from the blood stream by the salivary glands. Samples of arterial blood have been taken in addition to samples of mammary and jugular venous blood so that direct comparisons could be obtained. Lastly, the manometric method of Van Slyke [1929] has been used for the estimation of amino-acid-nitrogen directly in Folin-Wu blood-filtrates. This method has been found to give rapid and accurate results.

Experimental.

Blood samples were taken from lactating and non-lactating cows as previously described [Blackwood and Stirling, 1932, 2]. Duplicate amino-acid-nitrogen and urea determinations were carried out according to the manometric methods of Van Slyke [1927, 1929]. Iron was estimated iodimetrically [Blackwood and Stirling, 1932, 1]. Corpuscular volumes were estimated by centrifuging to constant volume in haematocrit tubes.

In Table I are given the results of a preliminary series of observations carried out to ascertain if the differences between jugular and mammary bloods in amino-acid-nitrogen content reported by Cary could be detected by these methods and established as normal findings.

Table I.

No. of cow	Lactating or non-lactating	Date of analysis	Urea-nitrogen; mg. N per 100 cc. of blood		Amino-acid-nitrogen: mg. N per 100 cc. of blood		Amino-acid-nitrogen: Difference. Jugular-mammary % jugular
			Jugular	Mammary	Jugular	Mammary	
1	Lactating	27. x. 30	12·12	12·93	7·036	6·739	4·2
2	,,	4. xi. 30	10·02	10·04	5·884	4·825	18·0
3	,,	10. xi. 30	7·192	6·969	6·420	5·768	10·1
4	,,	13. xi. 30	9·619	9·529	7·375	6·324	14·2
5	,,	17. xi. 30	9·72	9·04	7·257	6·276	13·5
6	,,	20. xi. 30	10·76	10·58	6·087	5·059	16·9
6	,,	26. x. 31	6·09	6·44	8·314	7·863	5·4
7	,,	24. xi. 30	11·49	11·31	7·593	6·145	19·0
7	,,	1. iii. 32	10·92	11·34	8·129	6·900	15·2
8	,,	27. xi. 30	13·57	13·475	7·551	6·792	10·0
9	,,	3. xii. 30	11·472	11·290	6·985	6·945	0·6
10	,,	8. xii. 30	9·53	9·709	7·290	6·210	14·8
10	,,	2. 11. 31	10·85	10·10	8·357	7·489	13·9
11	,,	15. xii. 30	11·51	11·69	7·695	6·857	10·9
12	,,	19. iii. 31	11·00	11·00	5·479	4·785	12·6
12	,,	14. ix. 31	10·08	9·24	7·554	6·754	15·9
2	Non-lactating	19. i. 31	8·01	7·83	5·771	5·790	− 0·3
12	,,	14. i. 31	9·95	9·77	5·922	5·934	− 0·2
12	,,	22. i. 31	10·20	9·77	5·949	5·934	0·2
13	,,	9. xi. 31	10·85	11·06	6·925	7·187	− 3·6
14	,,	23. xi. 31	5·46	6·34	6·556	6·643	− 1·3
15	,,	26. iii. 31	8·62	8·56	5·676	5·718	− 0·8

Table II gives the results of a second series in which complete analytical results (jugular, arterial and mammary) for eight cows were available. In this table the figures for jugular blood have been corrected for the influence of salivary secretion.

Table II.

No. of cow	Lactating or non-lactating	*Iron (mg. Fe per 100 cc. of blood)			Urea (mg. N per 100 cc. of blood)			Amino-acids (mg. N per 100 cc. of blood)			
		Jugular	Arterial	Mammary	Jugular	Arterial	Mammary	Jugular	Jugular (corr.)	Arterial	Mammary
1	Lactating	37·04	35·06	35·06	10·08	8·68	9·24	7·554	7·148	7·382	6·754
2	,,	38·75	37·71	37·33	9·59	9·87	10·99	8·549	8·235	9·438	8·170
3	,,	40·86	39·29	39·50	12·18	12·25	12·60	7·766	7·508	7·126	6·062
4	,,	36·69	35·14	34·26	10·36	10·57	10·85	6·241	5·977	5·475	4·813
5	Non-lactating	39·31	36·28	36·81	6·58	6·86	7·21	6·921	6·480	6·591	6·993
6	,,	34·74	—	32·94	11·41	11·03	11·34	7·653	7·257	7·848	7·653
7	,,	34·23	32·56	33·46	8·61	9·31	8·96	7·147	6·985	7·204	7·384
8	,,	37·28	34·60	34·89	11·74	11·20	11·74	6·413	6·003	6·101	6·388

* Iron determinations taken from Blackwood and Stirling [1932, 3].

In Table III the differences in the amino-acid-nitrogen contents of the blood samples are expressed as percentages of the arterial value for each animal. Table IV gives the results of a number of cases in which corpuscular volumes were available for the calculation of the amino-acid-nitrogen content of the corpuscles.

Table III.

No. of cow	Lactating or non-lactating	Differences in amino-acid-N content (as percentages arterial amino-acid-N)		
		Arterial-jugular	Arterial-mammary	Jugular-mammary
1	Lactating	3·16	8·49	5·33
2	,,	12·75	13·44	0·69
3	,,	− 5·36	14·93	20·29
4	,,	− 9·17	12·09	21·25
5	Non-lactating	1·68	− 6·1	− 7·78
6	,,	7·51	2·49	− 5·05
7	,,	3·04	− 2·5	− 5·54
8	,,	1·61	− 4·70	− 6·31

Table IV.

No. of cow	Lactating or non-lactating	Corpuscular volume		Amino-acid-N (blood) mg. N per 100 cc. of blood		Amino-acid-N (plasma) mg. N per 100 cc. of plasma		Amino-acid-N (corpuscles) mg. N per 100 cc. of corpuscles	
		Jugular	Mammary	Jugular	Mammary	Jugular	Mammary	Jugular	Mammary
1	Lactating	33·5	30·5	7·554	6·754	4·248	3·380	14·11	14·44
2	,,	37·0	36·0	7·766	6·062	4·808	2·242	12·80	12·85
3	,,	41·0	39·0	8·549	8·170	4·687	4·206	14·10	14·37
4	,,	39·0	37·0	6·241	4·813	3·224	1·419	10·96	10·59
5	,,	40·0	39·0	5·479	4·785	2·357	1·857	10·16	9·37
6	,,	43·0	42·5	8·314	7·863	3·714	2·601	14·41	14·71
7	Non-lactating	41·0	38·5	6·921	6·993	3·949	4·094	11·19	11·62
8	,,	36·5	35·5	7·653	7·731	4·584	4·658	12·99	13·31
9	,,	32·5	30·0	7·147	7·384	4·790	4·874	12·04	13·24
10	,,	38·3	34·8	6·413	6·388	4·056	3·978	10·21	11·00
11	,,	41·6	37·5	5·922	5·934	3·917	3·948	8·736	9·145
12	,,	40·0	39·0	5·949	5·934	3·819	3·857	9·154	9·202

Discussion.

Cary [1920] gives values for the amino-acid-nitrogen content of the jugular blood of cows which range from 3·99 to 5·34 mg. N per 100 cc. These are lower than the values quoted by Bock [1917] and Okada [1918] who give 7 mg. N per 100 cc. as an average. Allardyce *et al.* [1930] give 5–8 mg. N per 100 cc. of

blood, a range which is in close agreement with the values obtained in the present work (5·8–8·5 mg.). Hayden and Fish [1928] give 5·30–18·0 mg. N per 100 cc. of blood as the range of urea-nitrogen encountered by them in the analysis of the blood of dairy cows, while Allardyce *et al.* give 12–20 mg. N. The urea-nitrogen values obtained in this series range from 5·4 to 12·6 mg.

As will be seen from Table I the differences between the amino-acid-nitrogen contents of jugular and mammary blood are generally very considerable. In this respect the results are in agreement with those of Cary.

From Tables II and III it is seen that in the non-lactating animals the amino-acid values of jugular blood are slightly lower than those of the arterial blood, a result which might be expected to arise from the utilisation of amino-acids by the tissues of the head and neck. The mammary venous blood of the four non-lactating animals examined is, however, slightly richer in amino-acid-nitrogen than arterial blood. This fact can only be explained on the assumption that amino-acids are returned to the blood during its passage through the inactive gland. The following explanation may be advanced to account for this finding. In dairy cows there is usually a period of three months between the end of one lactation and the beginning of the next. At the end of the lactation period part of the udder tissue which, in high milking breeds, may be very extensive, is resorbed. After a period of rest the animal again begins to proliferate mammary tissue in preparation for the next lactation. As the observations on non-lactating animals have been carried out soon after the end of the lactation period, it may be that the observed differences in amino-acid content between arterial and mammary venous blood are due to the breakdown of tissue-protein in the udder, and the gradual re-entry of the amino-acids so formed into the circulation.

With lactating animals such a consideration is, of course, absent.

It will be seen from Table III that in each of the four lactating animals examined the amino-acid-nitrogen content of the mammary blood is markedly and consistently lower than that of the arterial blood. Only a small proportion of this absorbed nitrogen can be accounted for by the renewal of glandular tissue, so that the theory that milk-protein is synthesised from the free amino-acids of the blood appears to be firmly established.

The assumption that the amino-acid-nitrogen content of jugular blood is the same as that of arterial blood is not supported by the data now presented. In all the non-lactating animals, and in two of the lactating animals, it is lower, while in the remaining two lactating animals it is higher. No explanation can be advanced to interpret this latter fact, but it is quite clear that the amino-acid-nitrogen content of jugular blood cannot be taken as an accurate representation of that of arterial blood.

From the qualitative standpoint this may not be important, but where attempts are made to interpret the data quantitatively (as has, for instance, been done by Meigs [1922]), the observed differences between arterial and jugular bloods will invalidate any conclusions.

In this connection it seems appropriate to consider some data on the relative absorption of sugar and amino-acids from the blood-stream. In a previous paper it has been shown that different animals absorb different amounts of sugar from the blood-stream. In this paper it is shown that different animals absorb different amounts of amino-acid-nitrogen. It is of value therefore to discover whether the ratio glucose absorbed/amino-acid-N absorbed is the same for different animals. Differences in the sugar and amino-acid-nitrogen contents of the arterial and mammary venous blood of four lactating cows are available (Table V).

Table V.

No. of cow	Sugar absorbed from 100 cc. blood arterial-mammary mg. glucose	Amino-acid-N absorbed from 100 cc. blood arterial-mammary mg. N	$\frac{\text{Sugar}}{\text{Nitrogen}}$
1	5	0·628	7·9
2	16	1·268	12·6
3	8	1·064	7·5
4	10	0·662	15·0

From the above data it is seen that the ratio is not constant for different animals. It may then be expected that the sugar/nitrogen ratio in the milk from different animals will show variations which can be correlated with the above. That this is the case is shown in the figures 8·8, 9·5, 13·4, 15·6 obtained for the sugar/nitrogen ratios of the milk of four lactating cows taken at random from the same herd.

It has been noted above that although the amino-acid-nitrogen content of jugular blood cannot be taken to represent that of arterial blood in a strictly quantitative fashion, significant differences between jugular and mammary venous bloods may be accepted as indications of absorption. The procedure adopted in Table IV, where a comparison is made of the corpuscular amino-acid-nitrogen contents of jugular and mammary bloods, may justifiably be used to determine qualitatively whether the amino-acids of the corpuscles play any part in secretion. The fact that salivary secretion does not appear to affect the corpuscles in any way allows figures for jugular and mammary corpuscles to be compared directly without correction.

It will be seen that the ratio of amino-acid-nitrogen in the corpuscles to amino-acid-nitrogen in the plasma is approximately 3 to 1. No previous data on this point have been published, although Martens [1928] gives the figure of 3 or 4 to 1 for the human subject. In general, although relatively large differences are shown by the whole bloods and by the plasmas of lactating animals, only very small differences are shown by the corpuscles. In most cases, indeed, the amino-acid-nitrogen content of the mammary corpuscles is even higher than that of the jugular corpuscles. If the differences between the amino-acid contents of corpuscles and of plasma are due to indiffusibility (the only explanation which appears valid), we should expect *a priori* that

corpuscular amino-acids would normally play little or no part in direct exchanges between the blood and the mammary gland. That this is the case is amply borne out by the data presented.

SUMMARY.

1. In non-lactating cows the amino-acid-nitrogen content of the jugular blood is lower than that of arterial blood. The amino-acid-nitrogen content of mammary venous blood appears to be slightly higher than that of arterial blood, a fact which may be explained by the resorption of mammary tissue proteins during the post-lactation period.

2. In lactating cows the amino-acid-nitrogen content of jugular blood also differs from that of arterial blood. The amino-acid-nitrogen content of mammary venous blood is however always significantly lower than that of either jugular or arterial blood. This fact confirms the view that amino-acids are the precursors of milk protein.

3. It has been shown that the ratio of sugar to nitrogen absorbed by the mammary gland is different in different animals. The range of its variation is identical with that of the ratio sugar to nitrogen found in milk.

4. The ratio of corpuscular amino-acid-nitrogen to plasma amino-acid-nitrogen is approximately 3 : 1 for the dairy cow.

5. Evidence is brought forward which indicates that the amino-acids of the corpuscles play no part in the exchanges between the blood and the mammary gland.

In conclusion I have to thank Prof. E. P. Cathcart who initiated this work, and Drs N. C. Wright and J. D. Stirling for their helpful advice and criticism.

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XCIV. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

IV. ASPECTS OF THE PHOSPHORUS METABOLISM OF THE MAMMARY GLAND.

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The mammary gland appears to have the power of effecting molecular transformations involving phosphorus. Thus, phosphoric acid must be linked up with the amino-acids hydroxyglutamic acid, aminohydroxybutyric acid and serine to give the phosphopeptone which Rimington [1927] has shown to be the phosphorus-containing group of caseinogen. Again, the secretion of milk involves the production of insoluble, colloidal calcium phosphate, while Kay [1925] has found that 15–25 % of the total phosphorus of milk is in the form of acid-soluble compounds hydrolysed by phosphatases. That phosphorus is concerned in the secretion of fat is suggested by Meigs, Blatherwick and Cary [1919], who claim that phosphatides are absorbed from the blood-stream by the mammary gland, that the amount of phosphorus so obtained is greater than can be accounted for by all the phosphorus in milk and that the excess phosphorus is returned to the blood-stream in the form of inorganic phosphorus. These suggestions are based on data obtained by the application of the Kaufmann-Magne technique, a procedure which, as we have already shown [Blackwood and Stirling, 1932, 2, 3; Blackwood, 1932], involves certain unjustifiable assumptions. In view of the recognition of the important part played by acid-soluble phosphorus compounds in metabolism it appeared desirable to investigate more fully the nature of the exchange of phosphorus between the blood-stream and the mammary gland. In the present investigation we have undertaken the examination of the organic and inorganic acid-soluble phosphorus contents of arterial, jugular, and mammary venous bloods with a view to establishing (1) if jugular blood may be taken to represent mammary arterial blood in so far as phosphorus is concerned, and (2) if non-lipoid phosphorus is utilised by the mammary gland.

[1] Carnegie Research Scholar.

No. of cow
1
2
3
4
5
6
7
8

Experimental.

Samples of blood were taken simultaneously from the radial artery and from the jugular and mammary veins of lactating and non-lactating animals, as described in Part I of this series [Blackwood and Stirling, 1932, 2].

The samples were analysed for:

Iron content. This was determined iodimetrically (Blackwood and Stirling, 1932, 1).

Corpuscular volume. The haematocrit technique was used.

Total acid-soluble phosphorus and inorganic phosphorus. The colorimetric method of Fiske and Subbarow [1925] was used; organic acid-soluble phosphorus being estimated by difference.

Table I summarises the results obtained for four lactating and four non-lactating cows. In Table II the differences implicit in Table I are expressed as

Table I.

No. of cow	Lactating or non-lactating	Iron content, mg. Fe/100 cc. blood			Total acid-sol. P, mg. P/100 cc. blood			
		Jugular	Arterial	Mammary	Jugular	Jugular (corrected)	Arterial	Mammary
1	Lactating	42·32	39·34	38·95	9·60	8·93	10·60	9·70
2	,,	38·75	37·71	37·33	9·50	9·24	10·30	9·00
3	,,	40·86	39·29	39·50	11·60	11·15	12·10	11·44
4	,,	36·69	35·14	34·26	9·25	8·86	10·05	9·50
5	Non-lactating	39·31	36·28	36·81	9·10	8·40	9·70	9·60
6	,,	34·74	—	32·94	9·00	8·53	8·80	9·20
7	,,	34·23	32·56	33·46	9·90	9·41	11·00	10·60
8	,,	37·28	34·60	34·89	10·00	9·28	9·80	10·40

No. of cow	Lactating or non-lactating	Inorganic P, mg. P/100 cc. blood				Organic acid-sol. P, mg. P/100 cc. blood			
		Jugular	Jugular (corr.)	Arterial	Mammary	Jugular	Jugular (corr.)	Arterial	Mammary
1	Lactating	5·16	4·80	5·20	4·92	4·44	4·13	5·40	4·78
2	,,	5·26	5·12	5·48	5·34	4·24	4·13	4·82	3·66
3	,,	5·72	5·50	5·86	5·76	5·88	5·65	6·24	5·68
4	,,	4·44	4·25	4·72	4·70	4·81	4·64	5·33	4·80
5	Non-lactating	5·76	5·32	6·42	5·58	3·34	3·08	3·28	4·02
6	,,	4·70	4·46	4·76	4·90	4·30	4·09	4·04	4·30
7	,,	5·62	5·35	5·48	5·64	4·28	4·07	5·52	4·94
8	,,	5·82	5·40	5·86	5·62	4·18	3·88	3·94	4·78

Table II.

No. of cow	Lactating or non-lactating	Total acid-soluble P			Inorganic P			Organic acid-soluble P		
		Jugular-Mammary	Arterial-Jugular	Arterial-Mammary	Jugular-Mammary	Arterial-Jugular	Arterial-Mammary	Jugular-Mammary	Arterial-Jugular	Arterial-Mammary
1	Lactating	− 7·30	15·80	8·5	−2·20	7·60	5·4	−12·03	23·52	11·48
2	,,	2·32	10·29	12·62	−4·02	6·57	2·56	9·75	14·32	24·07
3	,,	− 2·40	7·85	5·45	−4·44	6·14	1·71	− 0·48	9·45	8·96
4	,,	− 6·40	11·9	5·5	−9·54	9·96	0·42	− 3·01	12·94	9·94
5	Non-lactating	−12·37	13·40	1·03	−4·05	17·14	13·08	−28·7	6·1	−22·56
6	,,	− 7·61	3·07	−4·54	−9·25	6·30	−2·94	− 5·14	− 1·22	− 6·36
7	,,	−10·8	14·4	3·6	−5·29	2·37	−2·92	−15·76	26·27	10·51
8	,,	−11·43	5·31	−6·12	−3·75	7·85	4·10	−22·84	1·52	−21·32

percentages of the arterial values. Table III shows the partition of acid-soluble phosphorus compounds between the plasma and the corpuscles in the jugular blood of lactating and non-lactating cows. Table IV gives similar data for the mammary blood.

Table III. *Partition of acid-soluble phosphorus compounds between plasma and corpuscles in jugular blood.*

	Blood			Plasma			Corpuscles			Org. acid-sol. P of corpuscles / Org. acid-sol. P of plasma
Corpuscular volume	Total acid-sol. P	Inorganic P	Org. acid-sol. P	Total acid-sol. P	Inorganic P	Org. acid-sol. P	Total acid-sol. P	Inorganic P	Org. acid-sol. P	
Lactating										
29·5	10·00	6·48	3·52	8·80	7·46	1·42	12·88	4·14	8·74	6·15
38·0	11·20	6·60	4·60	9·60	7·58	2·01	13·81	5·00	8·81	4·38
36·5	9·60	5·16	4·44	8·70	6·10	2·60	11·10	3·53	7·64	2·94
41·0	9·50	5·26	4·24	8·90	5·78	3·12	10·36	4·51	5·85	1·87
37·0	11·60	5·72	5·88	9·90	7·10	2·80	14·49	3·38	11·14	3·97
39·0	9·25	4·44	4·81	6·80	4·42	2·38	13·08	4·46	8·61	3·61
Mean	10·19	5·61	4·58	8·75	6·40	2·39	12·62	4·17	8·46	3·82
Non-lactating										
34·0	11·60	6·34	5·26	8·65	7·30	1·35	17·30	4·47	12·83	9·5
38·5	11·35	6·60	4·75	10·05	7·86	2·19	13·90	4·60	9·30	4·24
41·0	9·10	5·76	3·34	7·20	6·80	0·40	11·82	4·27	7·56	18·9
36·5	9·00	4·70	4·30	6·00	5·60	0·40	14·22	3·12	11·09	27·7
32·5	9·90	5·62	4·28	7·50	6·66	0·84	14·89	2·49	13·20	15·7
38·5	10·00	5·82	4·18	8·00	6·90	1·10	13·21	4·05	9·14	8·30
Mean	10·16	5·81	4·35	7·90	6·85	1·05	14·22	3·83	10·52	14·06

Table IV. *Partition of acid-soluble phosphorus compounds between plasma and corpuscles in mammary blood.*

	Blood			Plasma			Corpuscles			Org. acid-sol. P of corpuscles / Org. acid-sol P of plasma
Corpuscular volume	Total acid-sol. P	Inorganic P	Org. acid-sol. P	Total acid-sol. P	Inorganic P	Org. acid-sol. P	Total acid-sol. P	Inorganic P	Org. acid-sol. P	
Lactating										
28·5	10·10	6·56	3·54	9·0	6·56	1·44	12·88	4·13	8·75	6·07
36·0	10·8	6·3	4·5	9·0	6·8	2·2	14·00	5·30	8·70	3·95
33·5	9·70	4·92	4·78	8·20	5·68	2·52	12·69	3·40	9·26	3·67
39·0	9·00	5·34	3·66	9·00	6·38	2·62	9·00	3·72	5·28	2·01
36·0	11·44	5·76	5·68	8·96	6·40	2·56	15·83	4·61	11·22	4·38
37·0	9·50	4·70	4·80	7·00	5·34	1·66	13·76	3·54	10·22	6·15
Mean	10·09	5·59	4·49	8·52	6·19	2·17	13·03	4·11	8·90	4·37
Non-lactating										
33·0	11·60	6·40	5·20	8·70	7·30	1·35	17·50	4·45	13·05	9·7
38·5	10·95	6·66	4·29	10·00	8·06	1·94	12·47	4·42	8·05	4·15
38·5	9·60	5·58	4·02	8·70	6·96	1·74	11·04	3·38	7·80	4·48
35·5	9·20	4·90	4·30	7·20	6·18	1·02	13·04	2·82	10·28	10·07
30·0	10·60	5·64	4·94	8·70	6·82	1·88	15·03	2·90	12·00	6·38
34·8	10·40	5·62	4·78	8·66	7·08	1·58	13·73	2·87	10·79	6·90
Mean	10·39	5·80	4·59	8·66	7·07	1·58	13·80	3·47	10·33	6·95

Discussion.

Figures for the total acid-soluble phosphorus and inorganic phosphorus contents of cow's blood have been published by Hayden and Fish [1928] who give 5·70 mg. P per 100 cc. of blood and 9·29 mg. P per 100 cc. of blood as

inorganic and total acid-soluble phosphorus respectively. The mean values obtained by us for samples of jugular blood from twelve different cows are 5·71 mg. P per 100 cc. of blood as inorganic phosphorus and 10·18 mg. P per 100 cc. of blood as total acid-soluble phosphorus.

Discussing first the assumption of Meigs *et al.* [1919] that the phosphorus contents of arterial and jugular bloods are identical, reference to Tables I and II shows that this assumption is unjustified so far as acid-soluble phosphorus is concerned. In every case which we have examined we have found the acid-soluble phosphorus content of jugular blood to be lower than that of arterial blood. This results from a diminished content of both inorganic and organic acid-soluble phosphorus in jugular blood. It may therefore be assumed that, at the time when these blood samples were taken, both of these classes of phosphorus-containing compounds were being absorbed from the blood-stream by the tissues drained by the jugular vein. The qualification with regard to the time of observation is necessary because there cannot be continual retention of phosphorus, no matter how small the amount, by any tissue. The observations reported in this investigation were carried out on samples of blood taken 4 hours after milking and immediately after a meal. It is possible that, at such a period, the arterial blood is losing material to the tissues, a process which might be reversed at a later period in the day. In this connection Palmer *et al.* [1930] have reported large diurnal variations in the inorganic phosphorus content of the venous blood of dairy cattle. Such an observation is in accordance with a post-prandial period of absorption of phosphorus by the tissues followed by a period during which phosphorus is returned to the blood. Whether this interpretation be accepted or not, it is evident that the phosphorus content of jugular blood cannot be taken to represent that of arterial blood. If, then, any information on the phosphorus metabolism of the mammary gland is to be obtained from blood analysis it is apparent that analytical values for mammary blood must be compared with those for arterial blood.

It will be seen that the four lactating cows of Table I show smaller amounts of total acid-soluble phosphorus in mammary blood than in arterial blood. The difference appears to be due entirely to absorption of organic acid-soluble phosphorus compounds, since the amounts of inorganic phosphorus in the two bloods differ but slightly. For the four non-lactating animals both bloods have practically the same amounts of total acid-soluble phosphorus. With the exception of animal No. 5, for which an anomalous result was obtained, the inorganic phosphorus contents of both bloods are also equal. With the exception of one animal we have found that the organic acid-soluble phosphorus content of mammary blood is higher than that of arterial blood—an observation which was at first surprising. The work of Kay and Robison [1924], Martland and Robison [1926] and Lawaczeck [1924] has indicated, however, that there is probably a continual interchange of phosphorus between the inorganic and organic phosphorus compounds in the blood. It may be that as the blood proceeds further from the heart the ratio, organic acid-soluble

phosphorus/inorganic phosphorus, is increased, without change in the total amount of acid-soluble phosphorus present. Such a process would explain our finding that the organic acid-soluble phosphorus content of mammary blood is higher than that of arterial blood in non-lactating cows. If such be the case, the figures shown for the amounts of organic acid-soluble phosphorus absorbed by the active mammary gland are too low. Meigs *et al.* [1919] have stated that the active mammary gland returns excess inorganic phosphorus to the blood-stream, basing their conclusion on the finding that the inorganic phosphorus content of mammary plasma is higher than that of jugular plasma. Figures for whole blood are likely to give more accurate information on this point. In every case examined we have indeed found that mammary blood contains more inorganic phosphorus than jugular blood. This fact cannot, however, be related to the state of activity of the mammary gland in any way for the result was obtained in lactating and non-lactating animals alike. Moreover, if the mammary gland absorbs more phosphorus from the blood-stream than is required for the secretion of milk—a point which has not yet been demonstrated—we should expect to find more inorganic phosphorus in mammary blood than in arterial blood. In no lactating animal was this the case and we believe that the difference between jugular and mammary bloods in this respect results from the fact that the mammary gland does not absorb the inorganic phosphorus compounds of the blood-stream as do the tissues drained by the jugular vein.

Our results lead us to suggest that the mammary gland utilises the organic acid-soluble phosphorus compounds of the blood-stream, and the question arises as to the function which they perform in the secreting cells. Kay [1925] has found that the active mammary gland of the goat contains an enzyme capable of hydrolysing such phosphorus-containing substances. The secretion of milk must to a certain extent resemble bone formation, for both processes involve the separation of insoluble (or colloidal) calcium phosphate from soluble phosphorus compounds. Robison and his co-workers [Kay and Robison, 1924; Martland and Robison, 1926] consider that the calcium phosphate of bone arises by enzymic hydrolysis of substances of the nature of hexosephosphates and we suggest that there is strong presumptive evidence for a similar process taking place in the mammary gland. (*a*) The gland can absorb acid-soluble phosphorus compounds from the blood-stream; (*b*) it contains an enzyme capable of splitting such compounds; and (*c*) it secretes insoluble (colloidal) calcium phosphate. The evidence is of the same nature as that adduced for the intervention of acid-soluble organic phosphorus compounds in bone formation.

One of the difficulties of accepting Robison's theory of bone formation is that most of the acid-soluble organic phosphorus of the blood is carried in the cells. It is of interest therefore to consider some data on the distribution of acid-soluble phosphorus compounds between the corpuscles and the plasma of cow's blood. In the first place it is to be noted that the total acid-soluble phosphorus content of cow's blood is only about one-third of that of human

blood. This is accounted for almost entirely by the extremely low acid-soluble organic phosphorus content of cow's blood. In human blood, the plasma is almost devoid of organic acid-soluble phosphorus. From Tables III and IV it will be seen that the plasma of lactating cows contains appreciable quantities of organic acid-soluble phosphorus—amounts which, indeed, constitute a considerable fraction of that in whole blood. We have found the organic acid-soluble phosphorus contents of the jugular blood of lactating and non-lactating animals to be almost equal. The partition of this phosphorus between cells and plasma appears, however, to differ in the two states. In non-lactating animals there is about fourteen times as much organic acid-soluble in the corpuscles as in the plasma; in lactating animals there is only about four times as much. This would appear to indicate that during lactation there is a greater demand for organic acid-soluble phosphorus than in the non-lactating condition. In the former state a large proportion is carried in the plasma—a medium of transport which would facilitate exchanges with the mammary gland. The ratio is not so high in the mammary blood of lactating animals (Table IV) although it is still higher than that in non-lactating animals. This is as one would expect if the active mammary gland absorbs organic acid-soluble phosphorus. It would appear therefore that the acid-soluble phosphorus compounds of blood play an important part in the secretion of milk.

Summary.

The acid-soluble phosphorus content of the jugular blood of lactating and non-lactating cows is lower than that of arterial blood four hours after the ingestion of food. The lowering is shown both in organic and inorganic acid-soluble phosphorus.

The inorganic phosphorus content of mammary blood is higher than that of jugular blood and approximately equal to that of arterial blood in lactating and non-lactating cows. It is suggested that this is due to the uptake of inorganic phosphorus from the blood by the tissues drained by the jugular vein and to the absence of absorption of inorganic phosphorus by the mammary gland.

The organic acid-soluble phosphorus content of mammary venous blood is lower than that of arterial blood in lactating cows, but slightly higher in non-lactating cows. It is concluded that organic acid-soluble phosphorus compounds are utilised by the mammary gland during the secretion of milk. The suggestion is made that such compounds may be concerned in the secretion of calcium phosphate, thus bringing milk secretion into line with bone formation.

The partition of organic acid-soluble phosphorus between the corpuscles and the plasma is shown to be different for lactating and non-lactating animals. It is suggested that during lactation a greater proportion of organic acid-soluble phosphorus is carried in the plasma to meet the demand for such compounds during the secretion of milk.

We have to thank Dr N. C. Wright for his interest in this work, and Mr L. Jordan who obtained the blood samples.

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XCV. THE REDUCTION OF GLUTATHIONE BY A LIVER SYSTEM.

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Since the isolation of glutathione by Hopkins [1921] it has been commonly assumed that this substance acts as a catalyst of tissue respiration. This assumption is based upon its —SH content, its wide distribution, and its behaviour when added to washed tissue. More recently objections have been raised to this view owing to the fact that glutathione occurs in the tissues almost entirely in the reduced form and seems to be relatively inert. Thus Meldrum [1930] suggests that the constancy of the concentration of reduced glutathione in yeast is due not to its maintenance by reducing processes, but to the stability of the thiol form. Objections of this nature have been answered by the work of Hopkins and Elliott [1931] on the survival respiration of liver, if one may assume that the conditions of their experiments approximated to *in vivo* conditions. They have shown that when fresh chopped liver is suspended in Ringer's solution and shaken in air reduced glutathione is gradually oxidised. Thus in the case of rabbit-liver the reduced glutathione disappears after 6 to 8 hours' aeration at room temperature, while at 37° it disappears in 3 hours or less. They have conclusively proved that during this time reduction and oxidation processes are both proceeding, though the velocity of reduction gradually falls owing to the using up of the reducing substrate in the excised liver.

While glutathione may thus well function as a carrier of oxygen to reducing systems, it appears that it can be responsible for only a fraction of the total tissue respiration, and an estimation of its importance in this respect cannot be made until the nature of the systems involved in its reduction are known. This work was started with the intention of investigating the systems in liver responsible for the reduction. The only tissue system so far discovered to be capable of reducing glutathione is the "thermostable residue" of Hopkins and Dixon [1922], but it has been shown by Hopkins and Elliott [1931] that thermolabile systems also play an important part, since the reducing power of liver tissue is largely diminished by heating for 1 hour at 50°. Of thermolabile systems, most dehydrogenases have been tried, with negative results; the method usually employed being to wash the tissue thoroughly with water

and attempt to form a reducing system by the addition to it of such metabolites as lactic, citric or succinic acid, or hypoxanthine.

The work to be described here deals with a liver system which reduces glutathione.

Experimental.

In the experiments to be described the tissue generally employed was ox-liver, though sheep- and rabbit-livers were also occasionally used with similar results. The experiments usually consisted in incubating the washed tissue or tissue extracts with GSSG, in phosphate buffer at p_H 7·4 at 37°. The GSSG employed was prepared by oxidation of GSH with H_2O_2 [Pirie, 1931]. The amount of GSH produced during the course of the experiment was estimated either by the method of Tunnicliffe [1925] or of Kühnau [1931]. The reactions were carried out in 100 cc. flasks which were evacuated at the water-pump. In most cases toluene was added to the reaction mixtures. When it was not employed the experimental times were kept as short as possible, and the results were rejected if there was any obvious sign of bacterial growth.

Substrate in watery and alcoholic extracts.

There is no doubt that when minced liver tissue is repeatedly washed with water, its power of reducing glutathione is largely decreased, indicating that some part of the reducing system is washed out. This was the first point to be investigated.

Fresh ox-liver was minced and washed by shaking for 30 minutes with approximately twice its volume of distilled water. After centrifuging, the aqueous layer was removed, boiled, filtered, and stored *in vacuo* at 0°. The residual tissue was dried in a vacuum desiccator over sulphuric acid, and when dry was powdered, washed again with water and finally made up into a suspension with water. 10 cc. lots of this suspension were incubated overnight in phosphate buffer at p_H 7·4 with excess GSSG, with and without the addition of 10 cc. of the boiled watery extract. The amount of GSH formed was estimated by titration against $N/100$ iodine, with the following results:

	Increase in titre (cc. $N/100$ I)
Washed tissue + watery extract	3·28
Washed tissue alone	1·45

In a further experiment an extract of minced tissue was made with approximately 50 % alcohol. The filtrate was evaporated down to a small volume under reduced pressure and dried in a vacuum desiccator. The residue was taken up in a small volume of water for the purposes of the experiment. This concentrated extract was then incubated as before with a suspension of water-washed tissue. The results were as under:

	Increase in titre (cc. $N/100$ I)
Tissue + alcoholic extract ...	1·55
Tissue alone	0·31
Boiled tissue + alcoholic extract	0·20

It will be seen that while the tissue alone shows little activity, the addition of a boiled watery or 50 % alcoholic extract of tissue produces an active system. This was interpreted as indicating that the reducing substrate was soluble in water or 50 % alcohol. When the washed tissue suspension was boiled, addition of the extract failed to bring about a reduction of glutathione, showing that the system is thermolabile.

Enzyme in watery extract.

The capacity for reducing glutathione of a system produced by adding boiled watery extracts of liver to the washed tissue does not compare at all favourably with that of the unwashed tissue. A feasible explanation of this is that the enzyme responsible is itself water-soluble, and that much of it is lost in the watery extracts which are subsequently boiled. An attempt was made to prepare a cell-free preparation of the enzyme, based on the assumption that the latter was water-soluble.

Fresh ox-liver was minced, spread in thin layers in Petri dishes and rapidly dried in vacuum desiccators over sulphuric acid. The dried tissue was ground to a powder and extracted by grinding with water in a mortar for 2 to 3 hours, approximately 300 cc. of water being used for 50 g. of dried tissue. The mass was centrifuged, and the supernatant liquid was dialysed for 4 hours against a large volume of distilled water. On centrifuging, a clear red liquid was obtained, the activity of which for reducing GSSG was estimated by incubating it overnight in 10 cc. samples with oxidised glutathione, with and without the addition of a 50 % alcoholic extract of tissue. The following results were obtained:

	Increase in titre (cc. *N*/100 I)
Centrifugate alone	4·20
Centrifugate + alcoholic extract	4·65

The centrifugate thus showed a satisfactory degree of activity. It was further dialysed overnight and centrifuged again, and the clear liquid was saturated with ammonium sulphate. The precipitate was collected and dried. The yield from 50 g. of dried tissue was approximately 8 g. This preparation when combined with watery extracts of liver showed considerable activity in reducing GSSG, as is indicated by the following results obtained by incubating 0·25 g. of the precipitate with the extract overnight.

	Increase in titre (cc. *N*/100 I)
Enzyme preparation + watery extract	8·4
Enzyme preparation alone	0·3
Enzyme preparation + alcoholic extract	0·9

The alcoholic extract employed was made by extracting the dried tissue with 97 % alcohol, evaporating the extract to a small volume under reduced pressure, and finally drying in a vacuum desiccator. The residue was taken up in water for use.

It appears from the results that the reducing substrate, while being soluble in 50 % alcohol, is practically insoluble in 97 % alcohol.

The enzyme having been thus obtained in a reasonably stable cell-free form, an attempt was made to form a system capable of reducing GSSG by incubating it with metabolites likely to function as reducing substrates, such as lactic, citric, and succinic acids, glucose and hypoxanthine. A small but definite reduction was obtained with glucose, the concentration of this substance employed being 0·25 *M*; this, according to Harrison [1931], is the optimum for glucose dehydrogenase. It was at first thought that the low activity of the system might be due to the fact that when glutathione was used as the hydrogen acceptor the optimum concentration of glucose differed from that when methylene blue was used. Using glutathione as the hydrogen acceptor the concentration of glucose producing maximum velocity was found to be *M*/10, but even with this optimum concentration of glucose the rate of reduction compared very unfavourably with that obtained by the addition of boiled watery extract of liver to the enzyme. Thus, in a typical experiment of 4 hours' duration, using 0·25 g. of enzyme, the following results were obtained:

	Increase in titre (cc. *N*/100 I)
Enzyme + boiled watery extract	4·40
Enzyme + *M*/10 glucose ...	0·55

i.e. when boiled watery extract was used in place of glucose the reduction proceeded with 8 times the velocity. The almost complete absence of substrate from a 97 % alcoholic extract of liver also seemed to indicate that the glucose system could not be responsible for the reduction of glutathione by liver.

An attempt was next made to obtain some idea of the properties of the reducing substrate by a fractionation of tissue extracts. A start was made by taking advantage of the apparent insolubility of the substrate in alcohol.

50 g. of dried tissue were extracted with 300 cc. of water and the extract after boiling and filtering was evaporated under reduced pressure to a volume of approximately 50 cc. Ten times the volume of 97 % alcohol was then added, and after standing for 1 hour in the refrigerator the supernatant liquid was poured off, and the precipitate was washed with alcohol and dried. The supernatant liquid was evaporated to a small volume under reduced pressure and finally taken to dryness in a vacuum desiccator.

In view of the results previously obtained it was expected that the substrate would be found in the alcohol precipitate. However, on testing the fractions in the usual manner, the alcohol precipitate proved to have practically no activity, while the filtrate from the alcohol precipitation was appreciably active. When now both fractions were combined an activity was obtained which was much greater than would correspond to a summation of the two separate systems, and which approached that of the original watery extract.

	Increase in titre (cc. *N*/100 I)
Enzyme + alcohol precipitate + filtrate	7·05
Enzyme + filtrate	3·10
Enzyme + alcohol precipitate	0·55

Some substance contained in the alcohol precipitate is thus essential for the activity of the system.

The effect of this alcohol precipitate was then tried on the reduction of glutathione by glucose dehydrogenase prepared as above, and glucose. The results given below were obtained using 0·1 g. of enzyme with 0·1 g. of the alcohol precipitate in *M*/10 glucose. The duration of the experiment was 4 hours.

	Increase in titre (cc. *N*/100 I)
Enzyme + alcohol precipitate + glucose	2·80
Enzyme + glucose	0·25
Enzyme + alcohol precipitate	0·40

The alcohol precipitate is thus an activator of glucose dehydrogenase, and the complete system is capable of readily reducing glutathione.

The alcohol precipitate has a similar effect upon the reduction of methylene blue by glucose dehydrogenase. If the enzyme is prepared by half-saturation with ammonium sulphate instead of full saturation and is well dialysed before use, the enzyme and glucose alone show almost complete inability to reduce methylene blue. If however the activator is also present a rapid reduction is obtained. Thus in one experiment, using in each methylene blue tube 1 cc. *M* glucose, 1 cc. enzyme solution (4 %), 0·5 cc. phosphate buffer (p_H 7·4), 0·5 cc. 1/5000 methylene blue, with and without the addition of 1 cc. of activator (5 % solution), made up to a total volume of 4 cc., the following reduction times were obtained:

Enzyme + activator + glucose	9 mins.
Enzyme + activator ...	32 mins.
Enzyme + glucose	3 hours +

Qualitatively similar results were obtained at other p_H values.

It is thus certain that the alcohol precipitate contains a substance which is an essential part of the glucose dehydrogenase system. The nature of this factor has not yet been ascertained. The preparation also contains varying amounts of substances which are capable, in presence of the enzyme, of reducing both methylene blue and glutathione. It is necessary to fractionate the preparation and obtain the activator free from such substrates before its action can be demonstrated in an entirely satisfactory manner.

Discussion.

From the preceding results it is evident that glutathione is readily reduced by the glucose dehydrogenase system. It is however difficult to assess the quantitative importance of this system in the reduction of glutathione by liver. Hopkins and Elliott [1931] showed that the falling off of the reduction processes during the aeration of chopped liver was due to the using-up of the reducing substrate. In the course of such experiments, however, one would expect an increase rather than a decrease in the concentration of glucose, owing

to glycogen breakdown; and it thus appears that there must be systems in liver which reduce glutathione more actively than does the glucose dehydrogenase system. It seemed a possible alternative explanation that glucose dehydrogenase might have been destroyed during the vigorous shaking which takes place in the aeration experiments. However, attempts to test this point made by addition of the enzyme to excised liver during survival respiration failed to yield satisfactory results. In the course of the present work indications have not been lacking that there are other systems in liver capable of reducing glutathione. For instance, the activator as prepared by alcoholic precipitation of watery extract of liver is capable, when added to the enzyme preparation, of forming a system which in some cases actively reduces glutathione.

Further work on this problem is proceeding.

Summary.

1. Glutathione is reduced by glucose in presence of glucose dehydrogenase prepared from liver by a method similar to that of Harrison [1931].

2. The reaction proceeds slowly. It is greatly accelerated by an activator which can be extracted from liver. The activator is soluble in water but insoluble in alcohol.

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XCVI. AUTOXIDATION OF THE FATTY ACIDS. I. THE OXYGEN UPTAKE OF ELAIDIC, OLEIC AND STEARIC ACIDS.

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The various experiments carried out in the past to elucidate the nature of the absorption of oxygen gas by substances containing the ethylene linkage, more particularly when present in the unsaturated oils, have shown that this oxidation process yields somewhat complex products. In the case of the drying oils, which, when exposed to the air in thin films, absorb oxygen rapidly and become gelatinous, the complex nature of the products may be largely due to the existence of different fatty acids each containing two or more double bonds. In the case of a mono-ene, such as oleic acid, the oxygen uptake is almost negligible at ordinary temperatures, but the products of autoxidation should be less complex. It has been found possible greatly to accelerate the autoxidation of this acid and of its *trans*-isomeride elaidic acid, particularly by high dispersion of the acid, but also by the use of a catalyst and by moderate increase of temperature. It was found, however, that even stearic acid was liable to an appreciable autoxidation under these conditions if the temperature exceeded 75°. To avoid, as far as possible, any autoxidation other than that concerned with the ethylene linkage, it was necessary to work below this temperature.

In the earlier stages of the present work it was observed that when oleic acid was intimately mixed with about 1% of a catalyst in the form of cobaltous oleate, at summer temperatures oxygen was taken up by the highly dispersed acid, causing a considerable reduction in the iodine value after a few days' exposure. An idea of the oxygen uptake of this acid, partially purified, is indicated by some of the results given in Table I (p. 798). The product was an oil and was liable to be contaminated by impurities in the original acid. For this reason elaidic acid, the *trans*-isomeride of oleic acid was chosen for investigation. It can be obtained readily in a pure state and does not absorb oxygen when in its crystalline form, but when it is kept at temperatures slightly above its melting-point of 45°, appreciable absorption takes place, the rate depending as mentioned above upon dispersion, the catalytic agency and temperature. Roscoe and Schorlemmer [1890] observe that this acid will absorb oxygen when kept molten, and in about 14 days will change to a

yellow oil. It will be shown below that this period has been reduced to as many hours.

Slow oxidation exposes the first product or products to further change and so leads to possible complications. For this reason alone a high degree of dispersion of the acid was desirable, so as to bring the autoxidation into line with the oxidation reactions of solutions. Attempts to bring about the absorption of oxygen by elaidic acid in solution have been made. A closed oxygen system of measured volume was caused to circulate for some days through solutions of the acid in different solvents and containing about 0·05 % of cobaltous elaidate either dissolved or in suspension. When boiling solutions were used a simple method for circulating the oxygen was adopted. The vapour from the boiling solution was led to the top of a condenser. From the bottom the condensed vapour fell in small drops from a constriction. These drops fell through the vertical portion of a T-piece with a tube of small bore. A Sprengel action was set up, and oxygen gas, entering by the third arm of the T-piece, was carried in bubbles through the boiling solution. Unabsorbed oxygen eventually found its way round the condenser to the T-piece. A mechanical device for circulating the oxygen through cold solutions at room temperatures was also employed. In neither case was the rate of oxygen uptake comparable with that obtained by surface dispersion of the acid. Although the former methods are still being pursued, the latter have yielded the results which form the subject of the present communication.

Preparation of elaidic acid and the catalyst.

Fairly pure oleic acid was converted into its sodium salt, and from its solution the lead salt was precipitated, washed, dried and extracted with ether. The filtered ethereal solution was shaken with dilute hydrochloric acid until all the lead was removed, washed, dried and evaporated. The oleic acid so obtained was placed in a shallow dish in a desiccator, which contained sodium nitrite in place of the drying reagent. A little nitric acid was poured on to the nitrite and the lid of the desiccator closed firmly. The fumes of nitrous acid were readily absorbed, and in less than 24 hours the oleic acid changed to a solid mass. This was spread on absorbent paper to remove adhering oil and nitrous fumes and was recrystallised from dilute alcohol until it gave a sharp melting-point of 44·5°. Previous to weighing the acid was dried in a vacuum desiccator over sulphuric acid.

The catalytic action of cobalt, manganese, lead and iron as "dryers" in the paint industry has been found to apply to the autoxidation of elaidic acid. Comparative experiments show that cobalt is appreciably more active than are the other metals. A number of estimations of the oxygen absorbed for different forms of these various catalysts and for different periods will be described elsewhere.

In the recent experiments of this work, cobaltous elaidate has been used to the extent of not less than 0·1 g. of the salt to 20 g. of the acid, corre-

sponding to an introduction of about 0·05 % of the metal. The salt was prepared by precipitation, a solution of cobaltous chloride being added in equivalent quantity to that of a solution of sodium elaidate. The pale pink powder is anhydrous and readily soluble in alcohol to form a brown solution. It is slightly soluble in other organic solvents. It is incorporated with the acid by mixing ethereal solutions containing a little alcohol, or by dissolving the salt in the molten acid. The small proportion used imparts a faint brown coloration to the otherwise colourless molten acid.

The use of a large proportion of sand as the dispersing medium in more recent experiments may have introduced other catalytic agencies. Although acid-washed, the sand is never free from iron, which appears to exercise a pronounced catalytic action, at any rate after a period of exposure, during which, perhaps, the iron assumes a suitable soluble form.

The apparatus employed to bring about autoxidation.

A number of different forms of apparatus have been made use of. A convenient method for keeping the acid just above its melting-point and in the presence of a closed oxygen system was to introduce it on to the inner surface of a large flask, the surface being increased by the aid of powdered glass. The heat was supplied from a small electric candle lamp introduced into the centre of the flask, connecting wires passing through the rubber cork. A glass tube connected the oxygen in the flask with an outside supply, and a surrounding tin cylinder served to retain the heat and keep the acid in a molten state, the temperature adjustment being made by movement of a partial covering to the cylinder. With a 2-litre flask this method proved suitable for the autoxidation of 3–5 g. of the acid. The heat was not well distributed and a more evenly oxidised product was obtained by dispersion of the acid on filter-papers, loosely folded and made to fill a flask immersed in a water- or oil-bath. The application of these methods has provided a number of facts to be recorded, but the most valuable results have been obtained from the more recent experiments in which sand was used as the dispersing medium. About one part by weight of the acid was mixed with 80 parts of purified sand. It was calculated, by counting the number of grains in a small weight and assuming their shape to be near that of a hemisphere, that the surface area over which 10 g. of acid was dispersed approximated to 8 m.^{2} The acid, admixed with the catalyst as described above, was poured molten on to the sand contained in a dish over a water-bath. The acid and sand were thoroughly well mixed so that the latter had the appearance of an evenly distributed "dampness." After cooling the caked mass was powdered and introduced into the glass container in which the acid was to be oxidised. In some cases, when particular stress was laid on the determination of the rate of oxidation, a differential apparatus was employed, which corrects for variations in atmospheric conditions. It was introduced by Haldane [1920] and has been extensively used for measurements of oxygen uptake by a number of different

workers. It does not lend itself to the estimation of volatile products, nor to the removal of carbon dioxide liable to impede the rate of oxidation. The most satisfactory apparatus is one which includes a method of circulating oxygen through an oxidising chamber and through absorption tubes. The method adopted in the majority of the experiments to be referred to will be described briefly by reference to the accompanying diagram from which, for clearness, some details of the apparatus have been omitted. The glass cylinder *A*

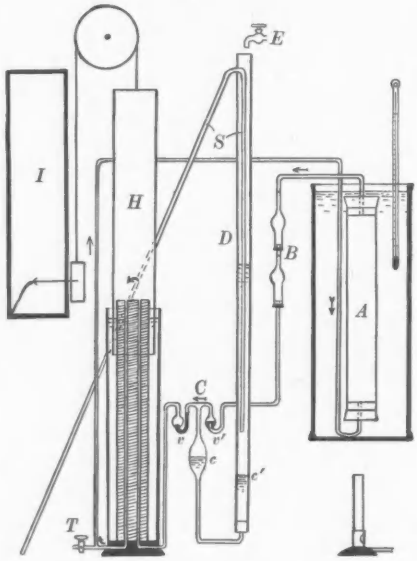

Fig. 1. Autoxidation of fatty acids.

A. Autoxidising cylinder immersed in water-bath.
B. Absorption apparatus for volatile products.
C. Valves.
D. Water column.
S. Intermittent syphon.
} Oxygen-circulating mechanism.
H. Oxygen gas holder.
I. Recording drum.

is of sufficient capacity to hold 800 g. of sand containing 10–20 g. of elaidic acid. The rubber corks are covered on their inner surfaces with tin foil. Layers of cotton-wool keep the sand from choking the inlet and outlet tubes at either end. This container is designed to ensure, as far as possible, an equal degree of oxidation throughout the mass. The oxygen and volatile products pass out by the upper small-bored tube and are led to the absorption tubes *B* containing calcium chloride and soda lime. The oxygen now passes to the circulating mechanism *C*. Two mercury valves *v* and *v'* will allow of gas passing only in the direction of the arrows. The mercury in the large bulb is given an

up and down motion by the water movements in *D*. The height of the water column in the wide tube *D* must be about 13·6 times the difference required in the mercury levels at *c*, *c'*. A water supply *E* flows continuously into *D*, which is emptied intermittently by the syphon tube *S*. The intermittent action, essential to the working of this circulator, is only possible when the bore of the syphon tube lies between 3 and 4 mm. Another necessary condition is that the bend of the syphon must not be constricted, but rather widened out. When *D* is emptying the syphon tube is full, when it is filling the inner portion of the syphon is a broken column of water, which on passing over the bend must flow down the slanting outer portion of the syphon as a continuous stream. This circulator if properly constructed will work continuously without any trouble. Temporary stoppage of water or slight variations in its pressure do not impede its efficiency. The oxygen is now forced into the holder *H*, which consists of a bell-jar moving up and down an annular space containing mercury. The mercury displacement is accurately compensated so that the holder delivers oxygen at the existing atmospheric pressure, and adjusts itself to all pressure changes. It is graduated to deliver known volumes of the gas. A T-piece at *T* enables the holder to be filled with oxygen as required, without interfering with the gas measurements involved in the experiment. This holder is described elsewhere [Ellis, 1931]. Finally the oxygen is returned to the bottom of the oxidising cylinder. A revolving drum *I* gives a graph of the oxygen uptake. The pen is attached to the weight balancing the bell-jar of the oxygen gas holder.

Over 40 different experiments have been undertaken with apparatus such as has been described above. Many of them were chiefly for the purpose of obtaining the oxidised product for chemical investigation but a number have furnished information on the rate of oxidation and on the amounts of oxygen uptake by elaidic, oleic and stearic acids. Table I contains the more important results obtained.

The rate of oxygen uptake.

In the absence of a catalyst, other than that afforded by absorbent paper, there is an induction period characteristic of the autoxidation of the drying oils. This period may last a day or two at temperatures below 55°, but above 70° it tends to disappear. Further, the induction period is lessened if the acid is heated for a short period to about 70° and then cooled to 55°. In an experiment particularly designed to show the difference of ordinary and catalytic autoxidation a differential apparatus comprising three bulbs was constructed. One of these bulbs acted as the control, a second contained 0·68 g. of elaidic acid dispersed on filter-paper. The third contained a similar quantity of acid and paper with the addition of the catalyst. Although at 62°, after 24 hours, the acid without the catalyst was only just commencing to oxidise, and in 3 days' time only 13·8 cc. of O_2 had been absorbed, the velocity increased with time and on the 4th day a volume nearly equal to that obtained for the previous 3 days was absorbed. In the case of the catalytic oxidation over

70 cc. were absorbed during the 1st day and the rate fell after this period. Without the actual addition of a catalyst the rate of the autoxidation of elaidic acid increases with time. It is therefore autocatalytic as has been observed in studies on the autoxidation of linseed oil [Genthe, 1906]. In the presence of a catalyst such as cobalt the rate falls from the commencement. This was shown in another experiment on 0·7186 g. in a differential apparatus. The actual hourly readings taken from the start, which represent the cc. of oxygen absorbed were: 12·2, 9·3, 8·4, 7·2, 6·4, 5·1, 4·2 and 3·4. After this period the fall was slow.

The experiments mentioned above, together with other similar ones, gave results only of value up to the periods given, beyond those periods the results were vitiated owing to the accumulation of carbon dioxide, which was not readily removed in the apparatus employed. In the experiments giving the results set out in Table I absorption tubes for removing volatile products and a circulating oxygen system as above described were employed. In recent experiments with the latter apparatus it has been the object to allow 20 g. of the acid to autoxidise as quickly, and at as low a temperature, as possible. By this weight of acid at about 74° an absorption of about 300 cc. of oxygen per hour can be procured. In all these experiments it was observed that there was never any definite cessation of oxidation. There was always a persistent absorption which, although less than 15 % of the maximum rate, was more in regularity and amount than could be accounted for by the decrease in the product of the originally reacting masses of what is probably a bimolecular reaction.

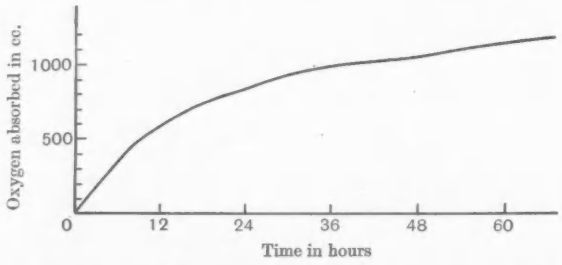

Fig. 2. Curve showing the uptake of oxygen by 5 g. of elaidic acid at 67°–70°. The ordinate represents cc. at room conditions (769 mm., 15·5°). Each atom of oxygen taken up per mol. of the acid is represented by a volume of 207 cc.

[*From a photograph of an actual curve obtained by the apparatus shown in the accompanying diagram.*]

The graph shown in Fig. 2, obtained as a result of Exp. 11 given in Table I, illustrates the above observations. In Part II it will be shown that a number of oxidation products are produced and that there is appreciable subsidiary oxidation to that which may be regarded as the main initial one.

The curve shown, although simple in its form, is that of a number of

processes, and shows, in particular, a high initial rate of autoxidation followed by a constant reaction velocity, for the latter part of the graph is a straight line except for a slight drop during the 46th hour. This was due to an accumulation of carbon dioxide following a temporary blockage in the soda lime absorption tube. From the results of other experiments extending for some days evidence goes to show that the autoxidation process or processes represented by the straight part of the graph will persist for many days. Oxidation that does not give rise to a reduction in the active mass is due probably to the gradual break-down of the fatty acid chain, and the continual evolution of water and carbon dioxide is in support of this view. It may be that this curve is a near representation of the biochemical oxidation of the fats in the animal organism.

The amount of oxygen absorbed.

Although there is an indefinite continuation in the autoxidation of elaidic acid, attempts have been made to judge of the amount of oxygen that might be concerned in an initial process. For this purpose the oxidation was brought about as rapidly as possible by high dispersion on sand and at a rate already intimated. It is to be realised, however, that the ethylene linkage is liable to produce an initial oxidation product highly susceptible to further change, and that even in the case of oxidation in solution, when both the acceptor and the oxidising reagent may be dispersed in molecular form, the amount of oxygen absorbed is generally in appreciable excess of that which may be required for an initial oxidation product.

In Table I the actual volumes of oxygen absorbed are set out in column 4 and in the next column the calculated percentages by weight of oxygen absorbed are given. The results of the first four experiments given are those obtained by deliberately stopping the oxidation before the marked decrease in the velocity of the reaction had taken place. It was possible to make an approximate determination of the unoxidised acid, and so arrive at the amount of oxygen absorbed, under conditions that would tend to eliminate any secondary oxidation process. The amounts of the unchanged acid, recovered in a crude form, are given in column 8. These results indicate that nearly 20 % by weight of oxygen was absorbed by the oxidised portion of the acid. It will be noticed that the periods set out in column 3 of Table I vary considerably for a given amount of oxidation. This variation is due to different degrees of dispersion of the acid and to different temperatures employed. A third cause may be variation in the effective incorporation of the catalyst. The results of Exp. 5 are in agreement with the above, for a small quantity of the unchanged acid was recovered. Exp. 6 shows the effect of oxidation at a slightly higher temperature. In Exp. 7 and the subsequent experiments traces of the unchanged acid were isolated, but oxidation had been allowed to proceed until a nearly constant, slow rate of oxygen uptake was reached. Exp. 9 shows the effect of prolonging the oxidation and Exp. 10 the effect of oxidation

Table I. *Oxygen uptake of fatty acids.*

No. of exp.	Weights taken g.	Duration and temperature	Observed oxygen absorbed cc. at N.T.P.	Percentage weight	CO_2 % by wt. as oxygen	H_2O % by wt. as oxygen	Remarks
			Elaidic acid.				
1	5·1348	12 hrs. at 63°	358	10·0	—	—	51 % }
2	8·1077	2 days at 60°	692	12·2	—	—	45 % } Impure elaidic
3	0·7186	6 hrs. at 67°	60·0	13·7	1·8	—	26 % } acid recovered
4	10·000	18 hrs. at 60°	1088	15·5	2·5	2·4	37 % }
5	10·050	36 hrs. at 65°	1198	17·0	3·5	3·2	
6	10·050	17 hrs. at 75°	1517	21·7	4·4	1·1	Higher dispersion
7	19·376	3 days at 60°	2748	20·3	3·6	—	—
8	20·000	5 days at 55°	3287	23·5	4·2	—	—
9	10·050	2 days at 65°	1484	21·2	4·0	3·0	—
		4 days at 65°	1824	26·0	5·3	4·7	—
10	6·000	3 days at 60°	784	18·7	3·2	5·6 }	No catalyst
		14 days at 60°	1224	29·2	5·5	12·1 }	
11	5·000	68 hrs. at 70°	1088	31·1	—	14·8	See graph
			Stearic acid.				
12	1·5558	11 days at 80°	63·9	16·4	5·5	5·2 }	Cobalt stearate as
13	0·2281	6 days at 75°	8·2	5·1	—	— }	catalyst
			Oleic acid.				
14	5·000	3 days at 14°	97	2·8	0·1	0·7 }	Two periods of one ex-
		2 days at 65°	692	19·8	6·3	3·4 }	periment. Impure acid with cobalt oleate as catalyst
15	10·091	23 hrs. at 37°	498	6·4	0·4	—	Fairly pure acid

in the absence of any other catalyst than may be afforded by the sand used for dispersion.

If 3 atoms of oxygen are concerned in the oxidation of each molecule of elaidic acid the uptake would amount to 17·0 %. If 4 atoms were so concerned a percentage of 22·7 would be expected. When the disturbing factors dealt with under the headings of the next two paragraphs have been considered, it will be realised that the lower percentage is all that can be expected to represent any initial oxidation process.

A comparative autoxidation of stearic acid.

It does not follow that any oxidation of elaidic acid, taking place elsewhere than at the ethylene linkage, would take place at a similar position in stearic acid. The comparison, however, is of interest. It was necessary to work at temperatures above 69°; otherwise the same technique was applied. At 75° and in the absence of a catalyst stearic acid did not take up oxygen in more than insignificant amounts, but in the presence of a small proportion of cobalt stearate the autoxidation became appreciable.

A comparison of the results obtained in Exps. 12 and 13 shows that there is a considerable difference in the rates of oxidation at the two temperatures 75° and 80°. At 80° the rate is comparable with that of elaidic acid, but it will be noticed that longer periods of exposure for both experiments were made. The nature of this oxidation has not been fully investigated. The products appear to represent a complex mixture, which is, however, largely

crystalline; and may lend itself to separation more readily than the products obtained by Salway and Williams [1922] and by Zorner [1923], who have oxidised the acid at 120–130° by passing oxygen through in the presence of manganese stearate. The large proportion of carbon dioxide and water evolved would indicate a degradation of the chain, and it must be supposed that this would happen even more so with elaidic acid under the same conditions. In the latter case however the lower temperature and much shorter periods employed have probably largely eliminated what would appear to be an extra autoxidation process to that concerned with the ethylene linkage. Further investigations of the autoxidation of stearic acid seem desirable. The results of such might throw further light on the nature of biochemical processes in fat metabolism.

The carbon dioxide and water formed.

In columns 6 and 7 of Table I are set out the approximate percentages of carbon dioxide and water formed. The figures represent the respective weights of oxygen contained in the two products, so that the sum of their weights deducted from the total oxygen uptake, as given in column 5, represents the weight absorbed by the main product. The results were obtained, as described above, by the continual circulation of oxygen through the system which included a calcium chloride tube and either potash bulbs or a soda lime tube. The circulation was continued for at least 24 hours after the autoxidation had been brought to a termination by removal of the source of heat. The figures obtained for water are only approximate owing to the presence in the absorption tube of volatile products. When sulphuric acid was used in the earlier experiments, this darkened considerably. That carbon dioxide was the main product absorbed by the potash bulbs was shown in the case of Exp. 9. The gas was liberated after its absorption and also from a control amount of the potash used: hence it was ascertained that at least 95 % of the increase in weight of the potash bulbs was due to this gas. In earlier experiments, before it was realised that appreciable volumes of this gas were being formed, the autoxidation was brought to an apparent cessation owing to its accumulation. If 1 atom of carbon were removed as carbon dioxide from a molecule of elaidic acid, the percentage of oxygen absorbed and evolved as this gas would amount to 11·3.

There is no doubt about the formation of quantities of water approximately represented by the figures shown; every precaution has been taken to ensure dry initial conditions. This product collects in the communicating tubes when the rate of autoxidation is at its height, and is only admixed with a small proportion of other volatile products. If from 1 molecule of the acid there was an evolution of 2 atoms of hydrogen as water, the oxygen concerned would represent 5·66 %.

Neither product seems to be present in an amount which points to its being concerned in the main initial autoxidation process, but both are formed in

appreciable quantities and may represent a degradation of the chain, comparable with that which stearic acid appears to undergo.

Deducting the oxygen evolved in these volatile decomposition products from the total absorbed, it is seen that 2 or 3 atoms are taken up by a molecule of the acid to form the non-volatile portion of the oxidation products. The results are lower than those obtained by Skellon [1931] by the autoxidation of pure oleic acid at considerably higher temperatures and for longer periods.

Summary.

1. Whereas oleic and elaidic acids do not absorb oxygen gas at ordinary temperatures except in negligible amounts, conditions have been devised under which autoxidation proceeds at such a rate as to cause an uptake by either acid of over 20 % of oxygen in from 24 to 48 hours. High dispersion of the acids is an important factor. Purified, dry sand has been employed as a successful dispersing medium. Autoxidation of elaidic acid in solutions of different solvents has not, as yet, been observed. The presence of about 0·1 % of the cobaltous salt of the acid provided an efficient catalyst, which has either reduced, or caused to disappear altogether, a more or less prolonged induction period. Warm conditions are essential. Below 55° the oxygen uptake by elaidic acid is slow. Above 75° stearic acid shows appreciable autoxidation under similar conditions of dispersion and catalytic agency. Hence oleic and elaidic acids have been allowed to autoxidise below 75° in order to avoid the possible introduction of an autoxidation process secondary to that concerned with the ethylene linkage.

2. Water and carbon dioxide are evolved during the autoxidation. In the case of elaidic acid, a number of determinations show that these are produced in amount considerably below that corresponding with a molecular proportion to the acid. Their formation appears to be due to secondary reactions, which continue indefinitely and probably represent a gradual degradation of the fatty acid chain.

3. The observations on the oxygen uptake of elaidic acid seem to show that under the most advantageous conditions for bringing about only an initial oxidation process, 3 to 4 atoms of oxygen are absorbed, and that 2 or 3 of these are retained by the non-volatile autoxidation products. The chemical nature of the latter will be dealt with in Part II.

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XCVII. A METHOD FOR THE ISOLATION OF GUANIDINE[1].

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(*Received March 21st, 1932.*)

A METHOD for the isolation of small amounts of guanidine in blood would be of interest in connection with several conditions, chiefly parathyroid tetany and diseases involving damage to the liver.

In the isolation of guanidine two main criticisms have been directed at methods previously put forward; (1) lack of separation of guanidine from creatine and creatinine and oxidation of the latter substances; (2) inadequate identification of guanidine salt. Later work has been confined to the estimation of a "guanidine substance" in blood by means of a colorimetric method. In all methods creatine and/or creatinine have been present when the final precipitation (or colour formation) was made.

In an endeavour to effect a complete separation of guanidine from creatine and creatinine, solutions of these substances were continuously extracted under reduced pressure with *iso*butyl alcohol, amyl alcohol, *n*-butyl acetate and toluene, but no separation occurred.

Whitehorn [1923] observed that treatment with sodium aluminium silicate (permutit) removed guanidine but left creatine and creatinine in solution. He makes the suggestion that all substances with a basic dissociation constant of 5×10^{-9} or greater are removed by permutit, while those substances with a smaller constant are left untouched.

Cannan and Shore [1928] state that the apparent dissociation constants for creatine and creatinine are $2{\cdot}9 \times 10^{-12}$ and $0{\cdot}4 \times 10^{-9}$ respectively, whilst guanidine has K_b $1{\cdot}1 \times 10^{-8}$ according to Whitehorn [1923] and $1{\cdot}4 \times 10^{-4}$ according to Morrell and Bellars [1907], the latter value appearing to be the more likely. If Whitehorn's suggestion is justified, then a separation of guanidine from creatine and creatinine seems possible, although arginine (K_b $1{\cdot}3 \times 10^{-5}$) and histidine (K_b $1{\cdot}0 \times 10^{-5}$) would be absorbed. Pfiffner and Myers [1926] state, however, that the absorption of guanidine and the methylated guanidines by permutit is not quantitative. I was unable to obtain a supply of permutit and used a similar preparation (doucil) procured

[1] The work contained in this paper formed part of a thesis which was accepted by the University of Durham for the degree of Ph.D.

from the British Drug Houses, Ltd. An analysis of doucil [Hilditch and Wheaton, 1925] is very similar to that of permutit in 1923 [Higgins and O'Callaghan, 1925].

	Na_2O	Al_2O_3	SiO_2
Doucil	13·2	21·8	65
Permutit	13·0	21·0	65

These approximate to Na_2O, Al_2O_3, $5SiO_2$.

Guanidine was found to be completely removed from neutral aqueous solutions by doucil, while no retention of creatine or creatinine could be observed.

For the removal of bases from the silicate, a saturated solution of sodium chloride was found to be most convenient. The amounts recovered were not quantitative, especially with dilute solutions of guanidine.

As would be expected from the balanced action suggested by Whitehorn, the removal of guanidine from saline solutions was very much less efficient than from watery solutions. The percentage recovery of guanidine dropped from about 60 % (and higher) in water, to about 20 % in 1 % saline. Thus it seemed clear that, although a separation of guanidine from creatine and creatinine could be achieved by means of "doucil," some removal of sodium and/or concentration of solution was desirable before this observation could be applied effectively.

The most hopeful method seemed to be by the use of an adsorbing agent, and charcoal, already used by other workers, was investigated.

Preliminary experiments showed that

(1) the greater the amount of guanidine to be adsorbed, the greater the amount of charcoal necessary:

(2) the efficiency of a given weight of charcoal is increased by increasing the strength of sodium hydroxide up to about 0·02 *N*:

(3) the efficiency of a given weight of charcoal is decreased by dispersion in a large bulk of fluid.

Thus the optimum amounts of charcoal and alkali depend on the volume of solution and the amount of guanidine. The amounts used in the final experiments were 4 g. of charcoal and 2 cc. of *N* NaOH for 100–250 cc. of fluid, when 20 mg. of guanidine base were adsorbed quantitatively.

The difference between these amounts and those used by Weber [1927] is accounted for by the fact that Weber was dealing with small amounts of fluid and low concentrations of guanidine, for which the amounts he gives are adequate.

For the release of guanidine from charcoal, the conditions used in conjunction with those given above for adsorption were 100 cc. of 0·1 *N* sulphuric acid in 95 % ethyl alcohol for every 4 g. of charcoal used.

In confirmation of the work of Pfiffner and Myers [1930] it was found that the recovery was incomplete, being only 75–80 %.

These two processes were then combined, and some experiments performed

in order to find if reasonable recoveries could be made of guanidine added to aqueous solutions. The percentage recoveries were as shown in Table I.

Table I.

mg. of base per 100 cc. water	% recovery
46·5 (guanidine)	42·5
22·5 „	45
1·4 „	20
0·7 „	20
0·2 „	Nil
57·5 (*as*-dimethylguanidine)	25
22·5 „	25
3·6 „	36
1·8 „	6
0·9 „	Nil

These figures, while not high, are about what would have been expected from the previous experiments, as the recovery from charcoal is about 75 %, and that from doucil of about the same order. With *as*-dimethylguanidine, which is (presumably) a much weaker base, the recovery is not so high.

In order to ensure that creatine and creatinine were not broken down during these manipulations, solutions containing 10 mg. of creatine, 10 mg. of creatinine, and 5 mg. of each substance per 100 cc. water were subjected to exactly the same procedure as the solutions of guanidine, but no trace of any breakdown could be shown.

Thus from aqueous solutions it is possible to isolate reasonably small amounts of guanidine bases without any breakdown of creatine or creatinine.

Precipitation and identification of guanidine.

The precipitating agents which have been used previously are salts of mercury, silver, gold and platinum, phosphotungstic acid, picric acid and picrolonic acid. Zimmermann [1930] found that guanidine and methylguanidine are precipitated in neutral or acid solution by quinizarinsulphonic acid (1:4-dihydroxyanthraquinone-2-sulphonic acid). This acid also precipitates a number of other substances amongst which are ammonia, creatinine, arginine, histidine and lysine. Smorodinzew [1930] attempted, without success, to separate methylguanidine and creatinine by the differing solubilities of their reineckates. Neither of these substances seems suitable in view of their complexity and probable difficulties of analysis.

Of the substances mentioned previously, the use of silver and mercury salts has been justly criticised by Ewins [1916] and by Greenwald [1919]. Gold chloride is apparently easily reduced. Burns [1916], who used it, found small amounts of gold in his precipitates, and Beilstein [1922] says that methylguanidine chloroaurate is easily decomposed. Although platinum chloride has been used as a precipitant for the guanidine bases, the salts formed are apparently not very insoluble. Beilstein [1922] gives the solubility of methylguanidine chloroplatinate as 14·3 parts per 100 parts of water at 18–19°. He also states [1922] that *as*-dimethylguanidine chloroplatinate is easily soluble

in water. Phosphotungstic acid gives a precipitate with guanidine which is fairly insoluble (200 mg. per 100 g. water [Drummond, 1918]), but is soluble in excess [Kuen, 1927]. Apparently the precipitates with phosphotungstic acid are quite amorphous.

Picric and picrolonic acids have been used by various workers in this connection, the former by Sharpe [1920] and Greenwald [1924], the latter by Kuen [1927], Komarow [1929] and Ellis [1928]. Either of these acids seems to be suitable, and both were investigated in order to determine which would be more efficient.

In all precipitations an equal volume of saturated sodium picrate, or sodium picrolonate, was added to the solution containing the base to be precipitated.

Table II gives the limits of precipitation of guanidine and *as*-dimethylguanidine under these conditions. Guanidine picrate is bright yellow, and the picrolonate forms very characteristic small red balls. Both the dimethylguanidine salts are pale yellow and show delay in precipitation.

Table II.

mg. base per cc.	Guanidine		*as*-Dimethylguanidine	
	Picrate	Picrolonate	Picrate	Picrolonate
1	Ppt. immediately	Ppt. immediately	Ppt. (2 hrs.)	Ppt. immediately
0·5	,,	Trace in 2 hrs. greater overnight	Ppt. overnight	,,
0·25	,,	Nil	Nil	Trace in 2 hrs. greater overnight
0·125	,,	Nil	Nil	Nil
0·0625	Ppt. on standing overnight	Nil	—	—

Under similar conditions a picrate is formed with 11·5 mg./cc. Na, 1·25 mg./cc. K, and 2·5 mg./cc. NH_4 (chlorides); the picrates of Mg and Ca are extremely soluble. Picrolonates are formed with 6 mg./cc. Na, 2·5 mg./cc. K, 2·5 mg./cc. NH_4 and 24 mg./cc. Mg. Calcium picrolonate is precipitated with 0·02 mg. Ca per cc., the solubility being less than that of calcium oxalate under similar conditions. The solubility of this salt has been investigated in detail, at the author's suggestion, by Robinson and Scott [1932].

Creatine (3 mg./cc.) and creatinine (0·75 mg./cc.) are precipitated by picric acid solutions, but neither substance appears to be precipitated by saturated sodium picrate solution.

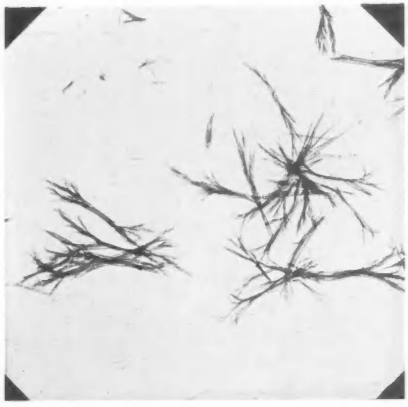
Photomicrographs of the guanidine derivatives are shown in Plates II and III. Photomicrographs of some of these compounds have been published previously by Burns [1916], Sharpe [1916, 1925].

Attempts were made to crystallise guanidine picrate from various solvents other than water but without success. Methyl, ethyl, *iso*butyl and amyl alcohol gave crystals which were not so definite in shape as those obtained with water. Butyl acetate, in which the picrate is very slightly soluble, gave poor crystalline form. In ether, chloroform, light petroleum and benzene,

PICRATES.

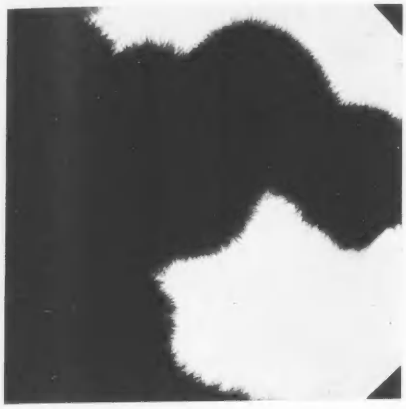
Guanidine picrate, × 340.

Methylguanidine picrate, × 60.

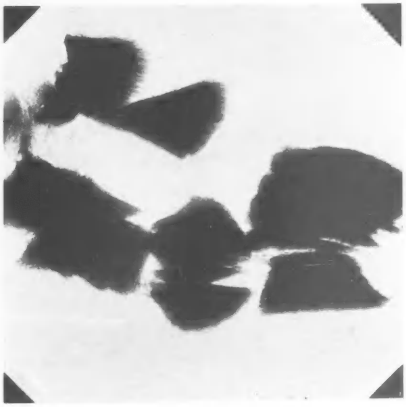


as-Dimethylguanidine picrate, × 60.

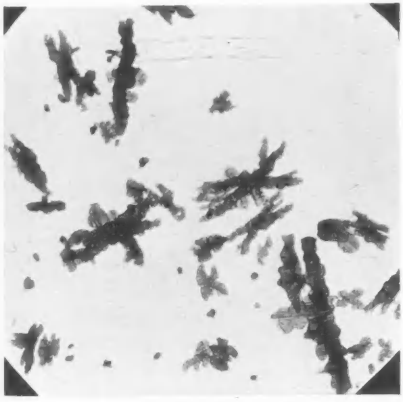
PICROLONATES.



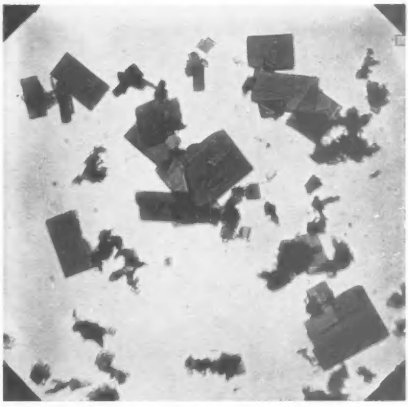
Guanidine picrolonate, × 210.



Guanidine picrolonate, × 210.



as-Methylguanidine picrolonate × 100



as-Dimethylguanidine picrolonate, × 210.

guanidine picrate is practically insoluble. The picrate is appreciably soluble in acetone, but the crystals obtained were very minute. Under these conditions, sodium picrate is in every way a better precipitating agent than sodium picrolonate. It is not only more efficient in precipitating guanidine, but is less easily precipitated by other bases.

Potassium and ammonia are the two most likely substances to be precipitated. The amounts of these substances in normal blood are [Harrison, 1930] K 150–250 mg. per 100 cc. and NH_3 0·1 to 0·25 mg. per 100 cc. Thus with normal bloods there is no likelihood of the precipitation of ammonia, but a distinct probability of the formation of potassium picrate. No way suggested itself of evading this difficulty excepting by analysis of the picrate.

In identifying picrates, methods used by previous workers have included melting-point, picric acid content, total nitrogen, and basic nitrogen [Greenwald, 1924; White, 1926–27]. Kuen [1927], using picrolonates, extracted the picrolonic acid with ether and weighed it. Of these the melting-point, basic nitrogen and picric acid content seem to be adequate. The estimation of total nitrogen is not suitable. It involves the use of a separate sample of picrate, and does not show such marked differences for different picrates as does the basic nitrogen. This latter can be estimated concurrently with the picric acid content.

Figures for the various picrates are given in Table III. As only small amounts of picrate would be available for analysis it is necessary to determine the smallest amount of picrate which would give a dependable result, and to ascertain the accuracy of the results.

Table III.

		M.P. ° C.	Basic N %	Picric acid %
Guanidine picrate		318–323 (G.) 317–320 (W.)	14·8	79·5
Methylguanidine picrate		197 (G. and W.)	13·9	75·8
Dimethylguanidine picrate		225 (G. and W.) 230 (B.)	13·3	72·5
Ammonium	,,	276–280 (G.) Volatilises (S. and P.)	5·7	93·1
Potassium	,,	311–316 explodes (S. and P.)	Nil	85·8
Sodium	,,	310–315 explodes (S. and P.)	Nil	91·2

G. = Greenwald [1924]. W. = White [1926–27].
S. and P. = Silberrad and Phillips [1908]. B. = Beilstein [1929].

The method of analysis adopted by Greenwald [1924] consisted in precipitating the picric acid, in a sample of guanidine picrate, with nitron, filtering through a Gooch crucible, and weighing the nitron picrate. The guanidine base in the filtrate he estimated by distilling with caustic soda, in a similar manner to the second stage of a Kjeldahl estimation. The smallest quantity on which a reasonably accurate analysis seemed feasible was 25 mg. From this amount of guanidine picrate, the weight of nitron picrate formed would be 47 mg., and the amount of ammonia distilled over would be

equivalent to 5·20 cc. of $N/20$ alkali. With a balance graduated to 0·0002 g., and a possible error of 0·0001 g. in each of four weighings (two in weighing the sample, and two in weighing the crucible) and each in the same direction, the difference would be 1·0 in the percentage of picric acid. In considering the estimation of the basic nitrogen, the calculation is not so simple, but by taking an actual example when 24·2 mg. of guanidine picrate were analysed, and calculating similarly the difference in the percentage nitrogen was 1·2. These calculations take a fairly extreme view of the probabilities of error, but such a view is necessary in endeavouring to determine the value of analytical figures. The differences in composition which would result from the admixture of 10 % of various picrates with pure guanidine picrate are given in Table IV.

Table IV.

Guanidine picrate mixed with	Difference in % basic N	Difference in % picric acid	Difference in M.P. of separate picrates
10 % potassium picrate	−1·5	+0·6	− 2°
10 % ammonium „	−0·9	+1·4	−137°
10 % sodium „	−1·5	+0·6	− 3°
10 % inert impurity	−1·5	−8·0	—

From these figures it seems that impurities would be easily attributed to errors in analysis, but that analysis would establish the identity of the substance forming the major portion of the picrate analysed.

This method was then applied to quantities of blood to which guanidine and *as*-dimethylguanidine salts had been added, protein being removed by the method of Folin and Wu [1919]. The amounts of guanidine recovered were lower than those from aqueous solutions, as will be seen from Table V.

Table V.

		mg. base added to 100 cc.	% recovery	
			Water	Blood
Guanidine	(1)	22·5	45	11
	(2)	46·5	42·5	23
as-Dimethylguanidine	(1)	22·5	25	Nil
	(2)	57	25	15·5

As control experiments two samples, each of 100 cc. of defibrinated blood, were treated in the same way as those to which guanidine had been added. No insoluble picrate was formed in either case.

The analytical figures for these picrates, together with those of guanidine picrate and *as*-dimethylguanidine picrate, are given in Table VI.

These picrates were not recrystallised before analysis, but in each case the small amounts left after analysis were recrystallised and showed the form characteristic of the picrate anticipated.

These figures show that a failure to find guanidine bases in blood by this method indicates that the concentration of these bases is less than 20 mg./100 cc.

of blood in the case of guanidine and 50 mg./100 cc. of blood in the case of *as*-dimethylguanidine, although it seems probable that smaller amounts would be detected.

Table VI.

		M.P. ° C.	Basic nitrogen %	Picric acid %
Guanidine picrate		317–323	14·8	79·5
,,	from water (1)	318 (decomposes)	—	—
,,	,, blood (1)	308 ,,	—	—
,,	,, water (2)	316 ,,	14·5	78·3
,,	,, blood (2)	306 ,,	15·8	77·0
as-Dimethylguanidine picrate		225	13·3	72·5
,,	from water (1)	227 (decomposes at 265)	—	—
,,	,, water (2)	219 (decomposes at 275)	13·6	70·6
,,	,, blood (2)	219 (decomposes at 260)	13·6	73·5

Experimental.

(1) *Extraction by partially miscible solvents under reduced pressure.* The apparatus introduced by Dakin [1920] was used. A solution containing 237 mg. creatine and 68 mg. guanidine in 250 cc. water was extracted with *iso*butyl alcohol for 36 hours. The extract contained 50 mg. creatine (20 %) and 17 mg. guanidine (25 %). Similar extractions were performed with amyl alcohol (which extracted a small percentage of guanidine), *n*-butyl acetate and toluene. The latter substances did not extract guanidine or creatine.

(2) *Experiments with doucil.* Doucil, a whitish granular solid, was kept in saturated sodium chloride solution (to remove calcium which was sometimes present). Before use it was washed thoroughly with distilled water and filled into a burette. A small reservoir was fitted to the top of the burette and the rate of flow of liquids regulated by an adjustable clip at the lower end. Prior to use the doucil was washed with dilute acetic acid (50–100 cc. of 1 %) and with water (200–300 cc.) until the filtrate was chloride-free and neutral to phenol red.

Solutions of guanidine were filtered through columns of doucil and followed by wash-water. In a few cases the filtrates were evaporated (with the addition of a little acid) to small bulk, and sodium picrate added. No precipitate resulted.

Attempts were made to remove guanidine from doucil by caustic soda and by alcohol, with no result. Washing with large volumes of water (400 cc.) gave no guanidine.

Guanidine was recovered from the doucil by washing with saturated sodium chloride solution, followed by an equal volume of water and, after evaporation to dryness, extracted from the sodium chloride with methyl alcohol, which is more efficient for this purpose than ethyl alcohol. The alcohol was evaporated and the residue extracted with methyl alcohol, which was again evaporated. The solid was taken up in water, but the solution contained a suspension (probably of silica) which could not be filtered or centrifuged. This was

removed by the addition of a little solid magnesium oxide, the solution being warmed to about 60°. A flocculent precipitate formed, which settled and was easily centrifuged. The clear liquid was decanted, neutralised, and an equal volume of saturated sodium picrate solution added.

Table VII.

Exp.	Fluid	Guanidine mg.	Doucil cc.	Saturated NaCl cc.	Recovery %
1	Water	20	34·5	50	57·5
				50	Tr.
2	,,	20	10	50	100
3	,,	10	13	50	40–50 (about)
4	,,	4·5	13	50	Tr.
5	,,	5	37	50	20–25
6	1 % saline	5	35	50	Nil
7	,,	69	15	50	20 (*a*)
8	,,	34·5	14	50	17·5 (*a*), (*b*)
9	,,	33·5	33	50	33 } 41
				50	8 }
				50	Nil
10	,,	28	14	50	11·5 p_H 8·2
				50	Nil
11	,,	29	14	50	14·5 p_H 5·5
		as-Dimethyl-guanidine mg.			
12	Water	22·5	12	55	68
13	,,	10	12	50	59
14	,,	6·5	11·5	50	Nil
15	,,	3	10	50	Nil

Volume of fluid, 500 cc.
(*a*) Solid sodium chloride re-extracted with methyl alcohol—no guanidine.
(*b*) Magnesium oxide (and silicate) re-extracted with water—no guanidine.

Solutions containing creatine and creatinine were filtered through doucil, and the doucil was washed with water. By estimations on the original fluid and filtrate, it was found that no creatine (or creatinine) was removed. The doucil was in each case extracted with saturated sodium chloride solution, but no insoluble picrate was formed.

Table VIII.

Volume of solution	mg. base (creatine)	Volume of filtrate	mg. base in filtrate
80	98·5	186	93
95	188	250	191
		300	Nil
	(creatinine)		
190	249·5	299	242
		100	2
		200	0·5
		100	Nil
95	247	250	249
		200	Nil

Volume of doucil = 20 cc.

(3) *Experiments with charcoal.* To neutral solutions containing guanidine were added caustic soda and charcoal (Merck's, pure, dry, "for analysis"). The mixture was shaken at intervals, and allowed to stand for at least 2 hours. The charcoal was then filtered off and allowed to drain. The filtrate was neutralised (the difference between the amount of acid needed and the caustic soda originally added, being taken as the amount of soda adsorbed), acidified and evaporated, the guanidine being precipitated as picrate. The charcoal and filter-paper were returned to the original flask, sulphuric acid-alcohol was added, and the mixture allowed to stand overnight. The charcoal was again filtered, water added to the filtrate and the alcohol distilled off on a water-bath. The liquid was then neutralised with caustic soda (or barium carbonate) and the guanidine precipitated with sodium picrate.

Experiments on the conditions of adsorption are given in Table IX.

Table IX.

Guanidine mg.	Volume of solution cc.	Normality of NaOH in solution	Charcoal g.	Guanidine in filtrate mg.	Adsorption %
4·6	100	0·016, 0·01, 0·005	1	Nil	100
23	,,	0·08	,,	8·2	64
,,	,,	0·04	,,	6·5	71
,,	,,	0·02	,,	6·2	73
,,	,,	0·01	,,	7·5	68
,,	,,	0·005	,,	9·5	59
10	,,	0·02	,,	Tr.	99
,,	,,	,,	2, 4	Nil	100
23	,,	,,	2	2	91
,,	,,	,,	4	Nil	100
20	,,	,,	,,	Nil	100
,,	250	,,	,,	Nil	100
,,	500	,,	,,	Tr.	99
,,	1000	,,	,,	1	95
,,	100	,,	,,	Nil	100
,,	250	0·008	,,	Nil	100
,,	500	0·004	,,	Tr.	99
,,	1000	0·002	,,	Tr.	99
23	100	0·02	,,	Nil	100 (14 times)
14	,,	,,	,,	Nil	100 (6 times)
20	,,	,,	,,	Nil	100
40	,,	,,	,,	1·5	96
80	,,	,,	,,	16	80

(4) *Release of guanidine from charcoal.* The volume of acid alcohol for efficient extraction varies with the amount of charcoal. With a constant amount of charcoal, the percentage extracted increases with the strength of acid and with the volume of the extracting fluid. The substitution of methyl for ethyl alcohol decreases the efficiency. The precipitation of sulphuric acid by barium does not decrease the yield compared with neutralisation by caustic soda (Table X).

(5) *Charcoal and doucil.* Solutions of guanidine (and dimethylguanidine) in water were treated with charcoal and then filtered through doucil. The yields are given in Table I. One illustrative experiment is given.

Table X.

Guanidine in charcoal mg.	Charcoal g.	Volume of acid alcohol cc.	Normality of acid	Guanidine released %	
16·5	1	50	0·02	52	
21	2	,,	,,	46	
23	4	,,	,,	20	
23·5	4	,,	0·33	69	
,,	,,	,,	0·17	67	
,,	,,	,,	0·08	59	
,,	,,	,,	0·04	60	
,,	,,	,,	0·04	51	
		(methyl alcohol)			
14	,,	50	0·1	58	neutralised with NaOH
,,	,,	,,	,,	62	,, $BaCO_3$ (cold)
,,	,,	,,	,,	58	,, $BaCO_3$ (heat)
,,	,,	100	,,	84	,, NaOH
,,	,,	150	,,	81	,, NaOH

95 % ethyl alcohol. 4 g. charcoal contain 1·7 cc. *N* NaOH.

46·5 mg. guanidine base (carbonate neutralised with H_2SO_4) in 100 cc. water were treated with 4 cc. *N* NaOH and 8 g. charcoal, and, after standing 2 hours, filtered. Guanidine was extracted by 175 cc. acid alcohol (= 150 cc. 0·1 *N* acid) overnight. The alcohol was evaporated and, after heating, 1·45 g. $BaCO_3$ were added. The solution was neutralised with 5·2 cc. *N* NaOH made up to 100 cc. and filtered through 26 cc. doucil in 2 hours, the doucil being washed with 200 cc. water. Guanidine was removed from the doucil by 50 cc. saturated NaCl solution (2 hours 20 minutes) and washed with 50 cc. water. The filtrate was evaporated to dryness on the boiling water-bath and the salt extracted three times with hot methyl alcohol (abs.) (total volume 45–50 cc.). The alcohol was evaporated and the residue again extracted three times with hot methyl alcohol (total volume 15–20 cc.). After a third evaporation the residue was dissolved in 15 cc. water (3 portions of 5 cc.): solid magnesia was added to the warmed solution until a flocculent precipitate formed which was centrifuged. The clear liquid was neutralised (1 drop *N* HCl) and 15 cc. saturated sodium picrate solution were added. After standing overnight the picrate was centrifuged, washed with 2 cc. water, dried at 100° and weighed.

Yield. 99 mg., 42·5 %.
Analysis. Basic N, 14·5; picric acid, 78·3 %.
M.P. 316° with decomposition. Microscopically—guanidine picrate.

Solutions of creatine and creatinine (100 mg. per litre) were treated in an identical manner but no insoluble picrate was formed.

(6) *Analysis.* The picrates were analysed in a manner similar to that used by Greenwald [1924]. At least 25 mg. of picrate were dissolved in 30 cc. water, and 10 cc. of 1 % nitron in 0·5 % acetic acid added, drop by drop, to the heated solution. The solution was cooled in the ice-chest and filtered through a weighed Gooch crucible. The precipitate was then washed with a little ice-water and the crucible dried in an oven at 100° and weighed. Nitron was

removed from the filtrate by the addition of 1 cc. of nitric acid to the boiling fluid, and cooling in ice with stirring for at least 2 hours. After filtration through a Gooch crucible, and washing with a little ice-water, the liquid was transferred (300 cc.) to the distillation flask of a Kjeldahl apparatus. 100 cc. of 40 % caustic soda were added, and the whole was distilled into standard acid, until the volume (in the flask) was 50 cc. 300 cc. of water were added and distilled over, when the whole of the base was broken down. When the distillation was repeated a third time no base came over. Melting-points were determined, using paraffin wax. All the readings given are corrected for cooling of the mercury thread. The M.P. of pure guanidine picrate was 320–323° with decomposition. If the heating of the picrate were very slow, there was a tendency for it to char with no apparent melting.

(7) *Removal of protein from blood.* Protein was removed by the method of Folin and Wu [1919]. By use of this method the resulting dilution is about eightfold, but attempts to avoid this dilution by precipitating proteins with alcohol and sulphuric acid-alcohol gave no good results. The incomplete filtration with Folin's newer method [1930] makes its use inadvisable.

The completed method was then as follows.

(*a*) *Removal of protein.* 1 volume of blood diluted to 8 volumes with water. 1 volume of 10 % sodium tungstate solution added. 1 volume 0·66 *N* sulphuric acid added slowly with shaking. Stand 1 or 2 hours and filter (usually overnight).

(*b*) *Adsorption with charcoal.* Neutralise with caustic soda, add 2 cc. *N* NaOH and 4 g. of charcoal for every 250 cc. of liquid (and, if guanidine has been added, for every 20 mg. of guanidine). Shake at intervals and stand for 2 hours. Filter.

(*c*) *Release from charcoal.* Add 100 cc. of acid-alcohol (0·1 *N* sulphuric acid in 95 % alcohol) for every 4 g. charcoal and shake. Stand overnight. Filter. Remove alcohol by evaporation on boiling water-bath. Heat to boiling and add, while boiling, slightly less than the calculated weight of barium carbonate. Stand and centrifuge off the barium sulphate. Neutralise carefully (using phenol red).

(*d*) *Removal of guanidine by doucil.* Filter slowly through 25 cc. doucil (previously washed until neutral and chloride-free). Wash doucil with 200 cc. water.

(*e*) *Removal of guanidine from doucil.* Filter 50 cc. of saturated sodium chloride solution, for every 20 mg. guanidine, slowly through the doucil, and wash with 50 cc. of water. Evaporate salt solution and wash-water to dryness on boiling water-bath. Extract solid sodium chloride three times with hot methyl alcohol (10–15 cc. each time). Evaporate methyl alcohol, and extract this salt three times with 5 cc. hot methyl alcohol. Evaporate off methyl alcohol. Dissolve residue in 15 cc. of hot water in a large centrifuge-tube. Put the tube in a boiling water-bath and add solid magnesia. When the liquid clears, cool and centrifuge.

(*f*) *Precipitation of guanidine.* Decant liquid into another centrifuge-tube, neutralise and add 15 cc. saturated sodium picrate solution, and stand overnight. If any precipitate is formed, centrifuge, wash with 2–3 cc. cold water and centrifuge again. Dry at 100° and weigh.

Details of the recoveries of guanidine from blood are given in Table XI. Analyses of the picrates are given in Table VI.

Table XI.

Base added to 100 cc. defibrinated blood	Vol. of filtrate cc.	*N* NaOH to neutralise cc.	Adsorption: *N* NaOH cc.	Adsorption: Charcoal g.	Release cc. acid-alcohol	Neutralisation: $BaCO_3$ g.	Neutralisation: *N* NaOH cc.	Vol. fluid cc.	Doucil: cc. (time)	Doucil: Saturated NaCl (time)	Vol. on adding picrate	Recovery
22·5 mg. guanidine	600	1·5	2	4	100	1	1·0	50	29 (1 hr.)	55 (1 hr.)	20	12 mg.–11 %*
46·5 mg. guanidine	750	0·6	14	20	400	3·36	5·7	100	25 (2 hrs. 15 mins.)	50 (2 hrs.)	15	54 mg.–23 %
										50	15	Nil
22 mg. dimethyl-guanidine	600	1·0	2	4	100	1	2·5	60	29 (1 hr. 20 mins.)	55 (45 mins.)	20	Nil
57 mg. dimethyl-guanidine	750	0·7	14	20	400	3·3	0·5	100	28 (2 hrs.)	50 (2 hrs.)	15	32 mg.–15·5 %
										50	15	Nil
Nil	600	2·5	2	4	100	1	2·5	50	27 (1 hr.)	55 (1 hr.)	20	Nil
Nil	750	0·7	14	20	400	3·3	0·7	100	29 (1 hr. 30 mins.)	55 (3 hrs.)	15	Nil
										50 (1 hr. 30 mins.	15	Nil

* MgO re-extracted—nil.

Summary.

A method is worked out for the estimation of guanidine bases in blood, particular attention being directed to the separation of these bases from creatine and creatinine.

An attempt to effect this separation was made by extraction with *iso*butyl alcohol, which extracts both substances partially, amyl alcohol, butyl acetate, and toluene, which do not extract either substance. The separation is effected by the use of doucil, a sodium aluminium silicate, which absorbs guanidine, but neither creatine nor creatinine. The removal of guanidine from doucil is incomplete, saturated sodium chloride solution being used, and the guanidine extracted from the salt by methyl alcohol. A preliminary concentration of the guanidine by adsorption on charcoal is found to be necessary, and the optimal conditions for adsorption and release of guanidine are worked out. Again adsorption is complete but release (by acid-alcohol) is only about 80 %.

The guanidine is precipitated by saturated sodium picrate and the solubilities of picrates of the commoner metals and guanidine are given, together with photomicrographs showing their crystalline appearance. The picrates are identified by melting-point, picric acid content and basic nitrogen.

With the completed method on aqueous solutions, 2 mg./100 cc. of guanidine or dimethylguanidine could be detected, although the recovery was only about 25 %.

For determinations in blood proteins were removed by the method of Folin and Wu. The percentage recovery from blood however dropped from 45 % (in water) to 25 % with 20 mg. guanidine per 100 cc.

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XCVIII. THE PRODUCTION OF VITAMIN D IN A GLOW DISCHARGE.

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(*Received May 4th, 1932.*)

Various workers have shown that vitamin D can be formed from ergosterol not merely by "ultra-violet light," but also by X-rays and cathode rays. We have, however, found no record of similar tests with a glow discharge, and we have therefore conducted a few experiments as described below.

The current used was 150 to 250 milliamp., alternating at 50 cycles from a transformer with a voltage of 2000. In the earlier tests ergosterol vapour at a temperature of about 130° and at a low pressure was passed through the whole discharge. As charring occurred round the electrodes, these were removed to side branches of the main (glass) tube so that the vapours passed through the positive column only. This procedure avoided the charring, but only gave a very small yield of vitamin D. It appeared possible that the formation of vitamin was due to ultra-violet radiation in the discharge rather than to electron bombardment. Accordingly, a 0·05 % solution of ergosterol in alcohol was passed at a rate of 16 cc. per min. through a silica tube of 5 mm. bore which traversed the centre of the positive column of the discharge for a length of 1 metre. This caused transformation of 18 % of the ergosterol to a resinous mixture containing a quantity of vitamin D (calciferol) equal to about 6 % of the original ergosterol. A similar test in which the glow discharge was passed through argon instead of air gave a destruction of 37 % of the ergosterol, and a mixture with an antirachitic activity corresponding to a yield of vitamin D equal to 16 % of the original ergosterol. In some tests the discharge tube was immersed in water at 0°. This caused a concentration of the "glow" to a cylindrical space closely surrounding the silica tube, but had little other effect. A similar test with a tube of greenish glass in place of the silica tube showed negligible destruction of the ergosterol, supporting the hypothesis that the action is due to radiation.

It is thus practicable to produce vitamin D in quantity in a glow discharge, but in our hands the yield per unit of electrical energy was much smaller than that obtained with a mercury vapour lamp.

XCIX. A NOTE ON THE ISOLATION OF ERGOTHIONEINE FROM BLOOD.

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(*Received April 7th, 1932.*)

The methods employed by Hunter and Eagles [1925], by Benedict, Newton and Behre [1926], and by Benedict and Newton [1929] for the isolation of ergothioneine from blood are somewhat involved and expensive to apply. The authors have devised a technique (based on Hopkins's [1929] method for glutathione) which yields about 85 % of the ergothioneine present. The method involves three stages.

(1) The extraction of the blood with dilute acid, and the subsequent coagulation and removal of the proteins.

(2) The concentration of the solution *in vacuo* and aeration of the concentrate.

(3) The precipitation and washing of the cuprous compound of ergothioneine, and its decomposition with H_2S, followed by concentration of the aqueous solution.

The whole process can conveniently be carried out in three days, and, if it be desired, glutathione can be recovered from the liquid filtered from the cuprous compound of ergothioneine.

Experimental.

5 litres of pig-blood were poured into an equal volume of dilute sulphuric acid (0·2 % by volume) and the whole warmed to 80°. The protein was filtered from the hot mixture on large Büchner funnels, the precipitate squeezed in a large filter press, and the expressed liquid added to the main volume of the solution. The volume of the total filtrate was about 8 litres. 1 litre of "dialysed iron" (B.D.H.) was added to the solution. The "dialysed iron" removes traces of protein and of haematin and appears to remove a so far unidentified substance, present in minute traces in pig-blood, which forms an insoluble compound with cuprous oxide. The mixture was filtered through pleated filter-papers and the filtrate evaporated *in vacuo* to a volume of about 1 litre and again filtered, to remove traces of uric acid.

The resulting solution contains reduced glutathione as well as ergothioneine. Reduced glutathione reacts with cuprous oxide, but oxidised glutathione does

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not [Hopkins, 1929]. The solution is brought to p_H 7·4–7·6 with alkali and vigorously aerated till the nitroprusside reaction for the sulphydryl group of glutathione disappears. (Ergothioneine does not give a nitroprusside reaction.) Sulphuric acid is added to 0·5 *N* concentration and the solution filtered, and warmed to 60°. A dilute aqueous suspension of cuprous oxide [Hopkins, 1929] is added in small amounts at a time, the liquid being vigorously stirred. The cuprous compound of ergothioneine falls out as a ropy precipitate which can be collected on a glass rod. Cuprous oxide is added till the precipitate acquires a faint purplish tint, but there is no danger in adding a slight excess of copper oxide. The cuprous compound of ergothioneine, unlike that of glutathione [Hopkins, 1929; Pirie, 1930], does not dissolve in presence of slight excess of cuprous oxide. The precipitate is allowed to settle and the supernatant liquid decanted off through a filter. The solution contains oxidised glutathione, which can be isolated by the usual methods. The precipitate of cuprous ergothioneine is then washed, best on the centrifuge with 60 % alcohol. About 10 washings are normally sufficient to remove sulphate. The copper derivative is then suspended in water and decomposed with H_2S. The copper sulphide must be well washed with hot water, and the combined filtrates are then evaporated *in vacuo* to about 10 cc. A few drops of concentrated hydrochloric acid are added, and the solution is further concentrated in a desiccator to 1 or 2 cc., when ergothioneine hydrochloride crystallises out, M.P. 208°. It may be converted to the free base (M.P. 262°) by the method of Benedict, Newton and Behre [1926]. (Found: N, 17·98; S, 13·91 %. $C_9H_{15}O_2N_3S$ requires: N, 18·34; S, 13·97 %.)

Hunter [1928] devised a modification of the Pauly diazo-test for histidine which, he claims, is specific for ergothioneine, and which permits of its quantitative estimation in blood. The total amount of ergothioneine was determined by Hunter's method. In general the yield of ergothioneine obtained by the above-described method was about 85 % of the total present.

SUMMARY.

A method is described of isolating ergothioneine from pig-blood. The yield is about 85 %, and the process is successful when the initial concentration of ergothioneine is as low as 20 mg. per litre and the earlier methods cannot be applied.

We have great pleasure in thanking Sir F. G. Hopkins for his interest in this work.

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C. THE REDUCTION OF GLUTATHIONE IN MAMMALIAN ERYTHROCYTES.

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GLUTATHIONE is found in most mammalian tissues [Hopkins, 1921; Blanchetière and Mélon, 1927]. Though it exists there in the reduced state as glutaminylcysteinylglycine [Pirie and Pinhey, 1929; Nicolet, 1930], little is known of the reducing mechanisms. Hopkins and Dixon [1922] studied a thermostable reducing system in muscle. They showed that an equilibrium existed between an insoluble factor in muscle called by them "fixed —SH" and soluble glutathione, according to the equation

$$G—S—S—G + 2TSH \rightleftharpoons 2G—SH + T—S—S—T$$

where —SH stands for sulphydryl and —S—S— for disulphide; T for tissue and G— for the glutathione residue. It is, however, clear that this affords but a partial explanation for the presence of reduced glutathione. The system is reversible, which means that the glutathione may reduce tissue disulphide, but it gives no explanation of the ultimate presence of sulphydryl. Some system, or systems, other than muscle protein must be able to reduce pentapeptide glutathione to the tripeptide form.

Hopkins and Dixon [1922] were unable to find any dehydrogenase system which, in presence of its substrate, reduced disulphide to sulphydryl, and the claim of Wieland and Bergel [1924] that succinate acted as a reductant in liver could not be substantiated by Elliott [1928]. Hopkins and Elliott [1931] found that liver systems could reduce added —S—S— to —SH and that the systems were thermolabile. They failed, however, to ascribe the reduction to any definite reducing system. Recently Mann [1932] has found that the glucose dehydrogenase of Harrison [1931] can reduce glutathione in the presence of glucose. This may be one of the systems responsible for the reduction observed by Hopkins and Elliott.

It has now become clear that glutathione behaves differently in different tissues. Hopkins and Elliott [1931] found that it disappeared rapidly from chopped liver (and also kidney) when the reducing metabolites had been exhausted by aeration. In marked contrast to their observations stand those of Meldrum [1930] who found no evidence for the participation of glutathione in the normal respiratory mechanism of yeast. As will be found in the following protocols, glutathione is remarkably stable to oxidation in erythrocytes.

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Though glutathione is found reduced in almost every cell system in which it exists, several factors militate against the identification of what reduces it. In yeast, for example, it does not become oxidised and yeast cannot absorb added G—S—S—G, which remains unaffected by the cell suspension. Treatment with acetone, as in making zymin, results in an apparent loss of reducing power. Washed chopped muscle appears to be unable to reduce disulphide glutathione in the presence of added glucose. When glutathione is not reduced the absence of effect may be due to (1) the impermeability of the cell-wall preventing access; or (2) the reducing mechanism having been destroyed by the artificiality of the conditions, or method of preparation.

Now Hopkins and Elliott [1931] found liver preparations to reduce added pentapeptide glutathione to sulphydryl, but were unable to find any familiar metabolite, which, when added to the mixture, accelerated or increased the reduction. In such a case the factor which prevents analysis is the complexity of the reacting mixture. The author has endeavoured to satisfy the conditions

(1) that the element of artificiality should be a minimum,

(2) that the reduction should be controlled by the presence or absence of some definite substance.

The mammalian erythrocyte has been found to satisfy all these conditions. When washed with saline and suspended in a salt solution of the appropriate composition it approximates in state to the natural conditions, and it is improbable that its metabolism differs much from that in the blood-stream. The red blood corpuscle contains reduced glutathione even when fully oxygenated, and (as will be shown later) the process of oxidation is comparatively slow and may be neglected in comparison with the observed rates of reduction in presence of hexoses.

No attempt has been made to attach functional significance to glutathione in corpuscles. Holden [1925] suggested that glutathione was concerned in erythrocytes with the reduction of oxyhaemoglobin and in exceptional circumstances with that of methaemoglobin (formed *in vivo* perhaps as the result of the presence of nitrites and other poisons). The results of Holden's experiments were due to a combination of two circumstances:

(*a*) that he used impure (*i.e.* non-crystalline) glutathione;

(*b*) that he used systems contaminated with iron or copper.

Turner [1929] has stated that the amount of reduced glutathione is 10–15 % greater in venous blood than in arterial, and his observations were to some extent confirmed by Gabbe [1928], who has, however, in his latest communication stated that his experiments were erroneous. It seems quite clear that glutathione cannot act as an oxygen carrier to any large extent in erythrocytes.

Experimental.

Section I. The interaction of cysteine and glutathione with oxyhaemoglobin and methaemoglobin was studied in a series of observations made as a preliminary step to the succeeding researches. Cysteine or glutathione was

allowed to react with protein in clean test-tubes and the system studied spectroscopically. For this purpose the following solutions were made:

(1) ox-oxyhaemoglobin was dialysed for about 8 hours in the apparatus of Stadie and Ross [1926] and diluted to a concentration of about 0·5 % with metal-free water;

(2) 10 % potassium ferricyanide was added to 5 % oxyhaemoglobin and the whole electrodialysed till free from ferricyanide in Stadie and Ross's apparatus;

(3) a solution of pure cysteine hydrochloride was brought to a p_H of 7·6 in phosphate buffer, the concentration being 4 mg. per cc.;

(4) crystalline glutathione was made up at p_H 7·6 in phosphate buffer, there being 8 mg. per cc.

1 cc. protein solution was added to 2 cc. phosphate buffer p_H 7·6 in a test-tube and 2 cc. of cysteine or glutathione solution. The reduction was followed spectroscopically. The results are shown in Table I. By a "rapid reduction" is meant a reduction in about 3 mins. or less. This is shown by a + sign. A − sign indicates no reduction.

Table I.

Reducing agent	Methaemoglobin	Oxyhaemoglobin
Cysteine	+	−
Cysteine + 10^{-2} mg. Cu ...	+	+
Glutathione	−	−
Glutathione + 10^{-2} mg. Cu	−	−

These observations are in accordance with those of Krebs [1929] on cysteine oxidation by haematin derivatives. It is of interest to note that the interaction between methaemoglobin and cysteine is direct and stoichiometric, whereas that between cysteine and oxyhaemoglobin in the presence of Cu depends on the dissociation of the oxyhaemoglobin when the dissolved oxygen has been used by the cysteine. There is no direct interaction between cysteine and oxyhaemoglobin.

The results of these observations show that *in vitro* glutathione and pure cysteine are unable to react rapidly with methaemoglobin and oxyhaemoglobin, and indicate with some probability that no such interaction takes place *in vivo* in the erythrocyte. It is thus improbable that the state of the haemoglobin system affects that of the glutathione *in vivo*.

Section II. The method of estimation of glutathione was based on that of Tunnicliffe [1925]. The corpuscles were lysed with an equal volume of water and a concentrated solution of trichloroacetic acid was added, the final concentration being 10 %. The protein was filtered, ground up with 10 % trichloroacetic acid, in volume equal to the initial amount of corpuscles, and filtered. The combined filtrates were titrated with $N/100$ I in the presence of 2·5 cc. of a 25 % solution of KI per 100 cc. filtrate, starch being used as indicator. The method has been much criticised because substances other than

glutathione, which absorb iodine, are present in tissue extracts and because the total iodine uptake of glutathione depends on the concentration and acidity of the solution and on the amount of KI present. The suggested changes appear to offer little real improvement, and the simple procedure has accordingly been followed. Table II shows the iodine uptake of crystalline glutathione prepared according to the method of Hopkins [1929] as modified by Pirie [1930] when titrated as in the experiments[1].

Table II.

Wt. of glutathione mg.	Observed titre cc. 0·01040 *N* I	Calculated titre* cc. 0·01040 *N* I
10	3·13	3·14
10	2·95	3·14

* Calculated on the assumption that the reaction is $2GSH + I_2 = GSSG + 2HI$.

When starch is used as indicator the end-point of the titration is somewhat difficult. Duplicate titres were however found to agree excellently. Table III shows the agreement found.

Table III.

No. of exp.	Vol. of corpuscle suspension (cc.)	Sample No. 1 titre cc. 0·01040 *N* I	Sample No. 2 titre cc. 0·01040 *N* I
1	25	1·38	1·36
2	50	3·25	3·18
3	50	3·26	3·20
4	50	2·18	2·05
5	50	3·47	3·18
6	50	2·85	2·79

These examples were chosen at random. It is clear that the method involves an error of not more than 10%, and, if experiment No. 5 be neglected, of 5%. Any increase of the iodine titre greater than 10 % of the total must be taken as indicative of a real change.

Ergothioneine (the trimethylbetaine of thiolhistidine) is present in blood-filtrates [Hunter and Eagles, 1925; Benedict, Newton and Behre, 1926], and titrates with iodine under the experimental conditions. Though the amount of ergothioneine in blood is dependent on dietary factors [Eagles and Vars, 1928] and varies much from species to species, the content of ox-blood appears to be consistently < 2 mg. per 100 cc. corpuscles [Hunter, 1928]. Ox-blood is peculiarly suitable for the investigations here described. Table IV shows the iodine uptake of ergothioneine when titrated with iodine under experimental conditions.

Table IV.

Wt. of ergothioneine mg.	Titre cc. 0·01040 *N* I	Estimated titre of ox-blood containing 20 mg. ergothioneine
20	3·8	220

The error introduced by the presence of ergothioneine is thus about 1·4 % and may be neglected.

[1] The iodine was standardised with other samples of crystalline glutathione.

Section III. The following experiments on the oxidation and reduction of glutathione in erythrocytes were carried out using corpuscles washed three times on the centrifuge with isotonic saline and suspended in isotonic sodium chloride. They were stored at 2° in an ice-chest. Such corpuscles contain much glutathione in the reduced state. This can be demonstrated by the intensely positive test for sulphydryl (ergothioneine does not react with sodium nitroprusside) and by the magnitude of the iodine titre. When such corpuscles are aërated at 25° or 37° a slow diminution occurs in the iodine titre and in the intensity of the nitroprusside test. Table V illustrates this effect.

Table V.

Exp. No.	Vol. of corpuscles cc.	Time of incubation hours	Titre cc. 0·01040 *N* I	Decrease %
A	50	0	2·67	0
			2·67	
	50	8·16	2·05	23
		8·16	2·18	
B	50	0	3·21	0
	50	6	2·1	34

These experiments were carried out with different samples of washed ox-corpuscles. The decreases in titre are much beyond the observed error. We have to regard glutathione in the erythrocyte as undergoing, certainly *in vitro* and probably also *in vivo*, a slow oxidation to the disulphide form. The steady state reached by the glutathione system in the red blood corpuscle depends on the relative rates of the two processes of oxidation and reduction.

Now the reduced form predominates in blood and the problem here studied is what reduces it. The reducing substances present in erythrocytes appear to consist (apart from glutathione) mainly of carbohydrates, and it seemed not improbable that they reduced glutathione, perhaps under the influence of some catalytic mechanism.

In order to test this possibility the following experiment was carried out.

Table VI.

No. of exp.	Conc. of glucose mg./100 cc.	Vol. of corpuscle suspension cc.	Time of aeration hours	Titre of experiment with glucose	Titre of experiment without glucose
1	150	50	0	1·63	1·63
			1	1·54	1·13
			15·5	2·39	0·64
2	300	50	16	3·57	0·75
3	100	25	0	1·37	—
			17	2·44	0·92
4	300	50	0	—	2·66
			2	3·00	—
			8·4	4·13	2·12
5	200	50	0	2·66	—
			3·9	3·00	—
			5·5	3·67	—
			8·25	4·13	2·05

Temp. of aeration was 37°.

Washed, aerated corpuscles were incubated with glucose at physiological concentration, and compared with similar corpuscles incubated in the absence of glucose. In each case the titre was found to be much greater with corpuscles incubated with glucose, and when most of the glutathione had originally been in the oxidised state the increase was as much as 300–400 %. The nitroprusside test was found to increase in intensity roughly in proportion to the iodine titre. Thus the disulphide glutathione had been reduced in the presence of glucose (Table VI).

Control experiments were carried out as follows. A 0·5 % solution of oxidised glutathione was made up in Ringer phosphate of p_H 7·6; 2 % glucose was made up in water, and one part boiled. The reaction took place in Thunberg tubes, evacuated and incubated at 37° for 12 hours. Experiments were done in duplicate. The protocols show the systems studied.

Table VII.

No. of Thunberg tube	1	2	3
Vol. of GSSG solution (cc.) ...	1	1	1
Glucose solution boiled (cc.) ...	0	0	2
Glucose solution unboiled (cc.) ...	0	2	0
Water (cc.)	2	0	0

No nitroprusside reaction could be observed in any tube after incubation overnight.

Under experimental conditions glucose alone cannot reduce oxidised glutathione.

From the data of Table VI it is clear that the reduction of oxidised glutathione existing in erythrocytes can be caused by the presence of glucose. When the corpuscles are lysed, by adding water to their suspension, or by the action of saponin, incubation with glucose is without effect on the amount of reduced glutathione. To reduce disulphide glutathione in the presence of glucose is a function of the intact corpuscle. This is shown in the following protocols.

Table VIII.

Time of aeration hours	Vol. of corpuscle suspension cc.	Amount of glucose mg.	Titre of intact corpuscles cc. N/100 I	Titre of lysed corpuscles
0	25	100	1·72	—
14	25	100	2·44	1·2

Lysis was caused by adding an equal volume of water to the undiluted corpuscle suspension.

When lysis was effected by adding 20 mg. saponin a similar result was obtained.

Table IX.

Time of aeration hours	Vol. of corpuscle suspension cc.	Wt. of glucose mg.	Titre of lysed corpuscles
9	25	100	1·6
9	25	0	1·5

These experiments were carried out on systems containing the glutathione which they normally possess. The lysed corpuscle system was further investigated with regard to its reducing action towards added glutathione.

25 cc. corpuscles were lysed with 20 cc. water in each of four 75 cc. Thunberg tubes. Oxidised glutathione was added and the whole incubated anaerobically at 37° with, and without, glucose for 6 hours. Had the added glutathione

Table X.

No. of Thunberg tube	1	2	3	4
Vol. of lysed corpuscles (cc.) ...	45	45	45	45
Vol. of 10 % glucose added (cc.)	1·25	1·25	0	0
GSSG solution (cc.)	0	2	0	2
Titre in cc. $N/100$ I	2·1	2·2	?	2·2

been reduced the extra titre in tubes 2 and 4 would have been 3·91 cc. The fact that the iodine titre was no greater in tube 4 than in 1 shows that the amount of the "fixed —SH" of Hopkins and Dixon [1922] in the intracellular proteins of the erythrocyte is inappreciable.

Warburg and Christian [1931] found that lysis of erythrocytes destroyed their respiration in the presence of glucose, but that it could be restored by adding Robison's hexosemonophosphate. No effect on the reduction of glutathione could be observed when hexosemonophosphate[1] was added to the lysed corpuscle suspension. This is shown in the following protocols.

Table XI.

No. of flask	1	2	3
Vol. of lysed suspension (cc.)	50	50	50
Glucose (mg.)	0	50	0
Hexosemonophosphate (mg.)	0	0	50
Titre in cc. 0·01040 N I ...	2·0	1·4	1·3

The corpuscles were incubated aerobically at 37° for 6 hours. The reducing effect of glucose on disulphide glutathione contained in the intact erythrocyte is likewise possessed by some other reducing sugars. This remarkable fact has been verified many times, invariably with positive results. On the other hand, pentoses were found to be almost devoid of reducing action, as were non-reducing sugars such as sucrose and raffinose, and the hexahydric alcohols sorbitol, mannitol and dulcitol. At first it seemed probable that the reducing action might be due to an impurity present in all the hexoses, perhaps glucose itself. As it is exceedingly hard to apply any simple test for the purity of a sugar the experiment was tried of measuring reduction as a function of the concentration of added sugar. When this was done it was found that the aldohexoses possessed the same reducing power towards glutathione whatever their chemical nature. Fructose possesses less reducing power than glucose. This seems effectively to dispose of the view that reduction with, say, galactose is due to the presence of an impurity common to it and all the other hexoses. It is

[1] A sample of hexosemonophosphate was very generously given me by Dr R. Robison.

scarcely credible that an unknown substance should be present to the same extent in glucose, galactose and mannose from widely different sources, and this view seems less likely than that the hexoses do actually reduce glutathione in the erythrocyte. The experiments with other sugars were carried out precisely as those with glucose. The suspensions of washed corpuscles were aerated at 37°, or exposed in a thin layer to clean air. The results with galactose are illustrated in Table XII, while the data obtained are summarised in Table XIII. In the experiments with galactose the reduction was studied by the use of the nitroprusside reagent. As with glucose the increase in iodine titre was found to follow the increase in comparative intensity of the nitroprusside test.

Table XII.

Vol. of corpuscle suspension cc.	Time of incubation hours	Amount of glucose mg.	Amount of galactose mg.	Titre in cc. *N*/100 I
25	0	0	0	1·37
25	16	0	0	0·92
25	16	50	0	2·44
25	16	0	50	2·51

It is evident that as regards net result the actions of glucose and galactose are similar.

Table XIII.

Substances which exert marked reduction	Substances which do not exert marked reduction
Glucose	Arabinose
Mannose	Xylose
Fructose	Sucrose
Galactose	Raffinose
Maltose	Mannitol
	Sorbitol
	Dulcitol

It was at first thought that the pentoses definitely exerted a slight reduction. This is always observed when the pentoses are used in concentrations of about 100 mg./100 cc. but tends to disappear when the concentration is about 20 mg./100 cc. It may thus be due to the presence of some impurity, and as the reduction was slight it is unlikely to be of biological importance, so the matter was not further investigated.

In order to study the reduction as a function of carbohydrate concentration a series of experiments was carried out, in which the cell suspension was incubated for an arbitrary suitable time with varying amounts of glucose. It was found that the reduction is independent of the amount of aldohexose within wide limits. *In vivo* this low limit can never be reached, except in hypoglycaemic convulsions of an extreme nature. The data obtained with glucose are set out in Table XIV and are plotted in Fig. 1.

In each vessel were 25 cc. corpuscle suspension, containing varying amounts of glucose. The suspensions were aerated for 9 hours at 37°.

Table XIV.

No. of vessel	1	2	3	4	5
Amount of glucose (mg.) ...	0	5	10	20	45
Titre of blood sample A ...	1·50	2·19	2·48	2·55	2·57
% increase in titre for sample A	0	46	66	73	73
Titre of blood sample B ...	1·26	1·98	—	2·26	2·24
% increase in titre for sample B	0	57	—	79	79

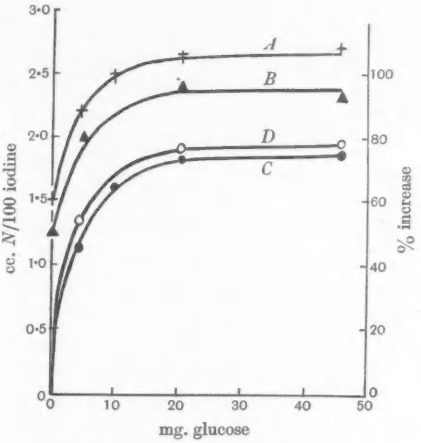

Fig. 1. *A*, Curve for blood A.
B, Curve for blood B.
C, % increase for blood A.
D, % increase for blood B.

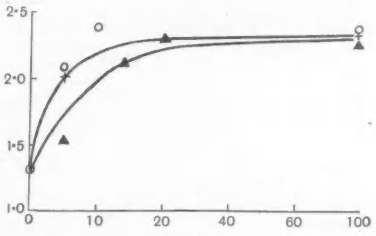

Fig. 2.

+ Glucose. ○ Galactose. ▲ Fructose.

The agreement is as good as can be expected between two different samples of blood.

Fig. 2 shows the comparative reductions shown by different carbohydrates plotted as a function of their concentration. The curves are not particularly favourable specimens, but all the experiments were carried out at the same time on one specimen of blood and for that reason are shown here. The work involved in obtaining such curves is very considerable and not many com-

parisons were made on such a scale. On the other hand many experiments were carried out using isolated points and the agreement was found to be close between the reducing powers of different hexoses.

In each vessel were 25 cc. corpuscle suspension, containing varying amounts of hexose. The suspensions were aerated for 9 hours at 37°.

Table XV.

Amount of hexose mg.	Titre with glucose cc. 0·01040 *N* I	Titre with galactose cc. 0·01040 *N* I	Titre with fructose cc. 0·01040 *N* I
0	1·31	1·32	—
5	2·02	2·08	1·55
10	2·33	—	—
15	—	2·09	—
20	—	2·26	—
100	2·25	2·28	2·23

The percentage increases in these experiments are smaller than those obtainable from the figures in Table VI. The discrepancy is more apparent than real: in the later experiments the corpuscles were used immediately after washing without preliminary aeration, so that less of the glutathione had become oxidised initially.

A considerable number of experiments were carried out in an attempt to link the reduction of glutathione with the glycolytic mechanisms of blood corpuscles described by Jost [1927]. It was found, however, that apart from lysis none of the factors such as fluorides which inhibit glycolysis exerts any marked influence on the reduction of glutathione, and no evidence could be obtained that it is in any way connected with these systems. The fact that hexoses other than glucose and fructose appear to reduce glutathione also militates against this view. Negative results were also obtained with cyanides and with phenylurethane. Neither of these appeared to influence the reduction.

Discussion.

It is extremely difficult to place the data obtained in the above experiments in any general scheme. The soluble muscle enzymes of Meyerhof [1926] appear to produce lactic acid from glucose, fructose, mannose and maltose, but not from galactose. The author is aware of no system in which glucose, mannose, galactose, fructose and maltose are attacked equally, other than that described above. The question as to whether there is any general biological function attached to the system described is at present quite obscure.

One point must be emphasised. The reduction of glutathione is a function of the intact corpuscle. Now, in many cases, though glutathione is everywhere found reduced, treatment of the system preliminary to its study seems to destroy the reducing system. Thus zymin did not reduce added glutathione when incubated under apparently favourable circumstances. Chopping brain tissue seems likewise to destroy its reducing activity: and so on. It may be

that the reduction of glutathione in these systems, as in erythrocytes, is intimately bound up with the intactness of the cell membranes.

Mann [1932] has recently described a water-soluble enzyme system from liver which reduces disulphide glutathione in the presence of added glucose. The system differs from the red blood corpuscle in that other hexoses appear to be inert, and there is no evidence that it can be extracted from other tissues. It is further questionable what fraction of the reducing power is exerted by this system in liver.

The observations here described offer but a very partial solution of the problem of the reduction of glutathione and it is impossible to do more than speculate on their general significance.

Summary.

1. In the intact erythrocyte the glutathione present undergoes a slow oxidation and a much more rapid reduction.

2. *In vitro* the reduction can be brought about by adding glucose to the washed suspension, and the increase can reach 300–400 %.

3. This reduction in the presence of glucose is shown only by intact corpuscles: lysis, with water or with saponin, destroys the reducing power.

4. Besides glucose other hexoses possess the power of reduction, as does maltose. Non-reducing sugars, and hexahydric alcohols derived from hexoses, cause no reduction, and in this way resemble the pentoses which reduce very feebly.

5. Curves are shown for the reduction as a function of carbohydrate concentration.

6. No evidence could be obtained that the system is in any simple way connected with the known glycolytic mechanisms of the erythrocyte.

I have very great pleasure in thanking Sir F. G. Hopkins for his interest in this work.

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CI. THE PROPORTION OF SKELETAL TISSUES IN INSECTS.

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The proportion of the skeleton to the weight of the whole insect might be needed for many purposes. My own reason for enquiring is that I have subjected insects to controlled atmospheric conditions, determining the loss of weight and the proportion of water in the insect before and after exposure. But when it has been demonstrated that under certain conditions the insect loses a particular amount of water and of dry material, it is not known how much of the remaining dry material is living matter on which the insect could perhaps call for energy, and how much of it is "dead" skeleton. It is the purpose of this note to discuss methods of estimating the weight of skeletal tissues in insects.

It is known that a considerable portion of the hard exo-skeleton of insects consists of substances other than chitin. Campbell [1929], for instance, has shown that in *Periplaneta* and several other insects the chitin makes up 30 to 40 % of the skeleton[1]. It is not known what the other materials are, though it is generally held that they consist (in part at least) of scleroproteins. For the moment it would suffice if an empirical figure for "skeleton" could be obtained. The proportion of chitin to skeleton differs in different parts of the same insect, and doubtless also in different species of insects. It is therefore not possible to estimate the chitin and deduce the total weight of skeleton from it.

Experiments with meal-worms.

It is clear that the proportion of skeleton can best be obtained by causing the soft parts of the insect to go into solution and estimating the residue. I have used digestive enzymes for dissolving the soft parts, and have also experimented with dilute alkalis and acid. The greater part of the work has been done with meal-worms (larvae of *Tenebrio molitor*, Coleoptera): nearly full grown larvae, weighing 60–190 mg., were used.

Enzymes. It seems clear that if a dried and powdered insect is treated successively with pepsin and pancreatin, what is left was not a part of the

[1] The proportion of skeleton in a certain batch of meal-worms was found to be about 8·3 % of the dry weight (below): the proportion of chitosan left after prolonged boiling in potassium hydroxide (60 g. KOH to 100 cc. water) was 5·05 %.

original living organs or tissues: in other words, the undigested portion is skeleton. But it is not certain that the whole of the skeleton is left, for some part of it may have been attacked by one or other of the enzymes.

I have carried out the work in the following manner. The insects are dried at 100° and reduced to a fine powder: it is necessary to break up the separate parts of the legs into fragments. If the proportion of fat is high, as it is in the meal-worm, it should be removed with ether. The material is then again dried at 100°, and a weighed quantity—say 200–500 mg.[1]—is transferred to a flask. In order to make the small fragments of chitin, *etc.* sink in the liquid containing the enzyme, they should first be moistened with acetone and then boiled in a few cc. of the buffer solution. A large volume—say 300 cc.—of pepsin buffered to p_H 2·5 is then added, and the material incubated at 37°, the reaction being adjusted daily if necessary. After 3 days the clear liquid is pipetted off and passed through a Gooch filter. The remainder is washed several times with distilled water, which is removed and passed through the same filter. The material is then digested with pancreatin at p_H 7·5 in the presence of toluene. After 3 days it is diluted with distilled water and passed through the same Gooch filter, which is then dried at 100° and reweighed.

From the results given in Table I, it seems that the method is accurate, for the meal-worm, to about 0·5 % of the dry weight (say 1·5 % of the original wet weight of the insect): for other insects the discrepancy is sometimes greater. It appears to be immaterial which enzyme is used first. It seems also that an exposure of 3–4 days to each enzyme at 37° is sufficient. A still longer exposure does not materially alter the result, from which I argue that the enzymes do not attack the non-chitinous material in the skeleton. This may readily be shown by taking the Gooch filter containing the solid remains and standing it in a beaker of pepsin or pancreatin for several additional days. At the end of that time there is generally a very slight reduction in weight. The *Tenebrio* material, which was treated with pepsin for 4 days and pancreatin for 2, contained 8·10 % of skeleton. Further treatment for 2 days with pepsin and 4 with pancreatin only reduced the percentage to 7·98.

Acid and alkali. It would be a much simpler procedure if the dried material could be treated with a dilute acid or alkali, and a result obtained consistent with that given above. The results of a series of experiments with 1 % hydrochloric acid, 1 % potassium hydroxide in water, and 5 % potassium hydroxide in absolute alcohol are shown in Table I. It is clear that treatment with 1 % KOH, at 100°, gives a result consistent with that obtained by the use of enzymes. The results obtained by the other methods are inconsistent. The 1 % KOH at 100° gives a result in 4 hours, and the proportion of "skeleton" is not reduced if treatment be continued for 24 hours. This method, therefore,

[1] It would be easy to work with greater quantities of meal-worm and obtain rather more accurate results. But it is essential with many insects to work with great economy. It must be remembered that many insects weigh less than 10 mg. (of which about two-thirds is water), and that it is often impracticable to obtain large numbers.

Table I. *Skeleton left after treating dried powdered meal-worm with enzymes, and dilute acids and alkalis.*

The material had been previously freed from fat with ether, but the results are expressed as percentages of the total dry weight.

ENZYMES.

Pepsin (days)	Pancreatin (days)	Skeleton %	Pancreatin (days)	Pepsin (days)	Skeleton %
1	0	19·8	3	0	10·1
4	0	14·3	5	0	9·5
4	1	10·1	1	2	12·2
4	2	8·1	1	4	8·35
			3	4	8·7

ACID AND ALKALI.

Reagent	Temp. ° C.	Time (hrs.)	Skeleton %
1 % HCl, aqueous	100	4	40
,,	,,	24	22
,,	,,	72	13
5 % KOH, abs. alc.	100	1	7·4
,,	,,	3	6·4
,,	,,	24	6·4
,,	,,	48	5·1
1 % KOH, aqueous	100	4	8·2
,,	,,	24	8·3

is the best to use, if it can be shown with other insects that the enzyme and potash methods give consistent results, and that an end-point is reached in a definite period.

RESULTS OBTAINED WITH OTHER INSECTS.

The dried powdered remains of other insects, freed from fat when necessary, have been subjected to digestive enzymes, and to 1 % KOH at 100°, by the methods already described. The results are presented in Table II. The *Periplaneta americana* (Blattidae, Orthopt.) were adults of both sexes. The *Culex pipiens* (Culicidae, Dipt.) were hibernating females, examined in November. The *Lucilia sericata* (Muscidae, Dipt.) were adults under 48 hours old. The *Rhodnius prolixus* (Reduviidae, Hemipt.) were adult males under 24 hours old. The *Cimex lectularius* (Cimicidae, Hemipt.) were adults of unknown age fed 10 days before.

Table II. *The proportion of "skeleton" in certain insects as determined by the enzyme method and by digesting in 1 % aqueous KOH at 100° for 24 hours.*

Name	Mean live weight mg.	Weight % (dry = 100)			
		Live	Fat	Skeleton by enzymes	Skeleton by KOH
Tenebrio (larv.)	120·0	242	36·4	8·1– 8·7	8·2–8·3
Periplaneta	960·0	298	6·2	15·1–16·7	11·6
Culex	2·63	224	47·0	11·6–12·7	13·0
Lucilia	26·4	329	Trace	19·4–20·0	—
Rhodnius	110·0	340	Trace	11·8–12·2	10·8–11·2
Cimex	4·0	330	Trace	36·0	17·5

With several of these insects I was able to show that either method reduces the amount of insoluble material to a definite point. For instance *Rhodnius*, after 4 days in pepsin and 1 in pancreatin, left a residue of 12·5 %; 2 more days in pancreatin reduced the figure to 11·8 %; after 3 further days in pepsin it remained unaltered. Samples of the same material treated with potassium hydroxide gave 11·05 % after 3 hours, and 11·2 % after 24 hours.

With each species of insect there is a general agreement between the results obtained with enzymes and with potassium hydroxide, having regard to the small quantities used. The figures for *Rhodnius* and *Cimex* show less residue by the potassium hydroxide method. This is probably due to the fact that the insects contained haematin, the remains of a previous meal on blood. This is not attacked by the enzymes, and is therefore reckoned as skeleton when they are used; but in the 1 % KOH it passes quickly into solution.

Summary.

It is found that if dried powdered meal-worm is digested with pepsin and pancreatin, the reaction stops or becomes very slow when 8·1–8·7 % of the original dry material is left. The same proportion (8·3 %) of residue is left if the material is exposed to 1 % aqueous KOH at 100° for 24 hours, and here again the reaction reaches an end-point. When the same methods are applied to other insects, they also give consistent results.

The potassium hydroxide method is simpler and quicker, and has the additional advantage (in dealing with blood-sucking insects) of dissolving haematin.

REFERENCE.

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CII. THE BIOCHEMISTRY OF ALUMINIUM.

III. EFFECT OF ALUMINIUM ON GROWTH AND REPRODUCTION IN THE RAT.

IV. THE OCCURRENCE OF ALUMINIUM IN THE THYROID.

V. INTESTINAL ABSORPTION OF ALUMINIUM IN THE RABBIT.

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III. EFFECT OF ALUMINIUM ON GROWTH AND REPRODUCTION IN THE RAT.

The use of rats for testing the biological importance or significance of foodstuffs and accessory factors is of comparatively recent introduction. This paper deals with the effect of aluminium in the rat when various essential constituents of the diet are varied, as well as with its effect when added to normal diets.

Other workers who have tested the effect of aluminium on rats include Myers and Mull [1928], McCollum, Rask and Becker [1928], Rose and Catherwood [1929–30] and Massatch [1930]. In all these experiments, aluminium added to the ration in amounts up to 0·1 % produced no effect when the diet was otherwise adequate. Lyman and Scott [1930] reported that no change in blood constituents (non-protein nitrogen, *etc.*) occurred on such diets. Kraft [1930] stated that the digestibility of food was not impaired by addition of aluminium. *Post mortem* examinations performed by most of these workers indicated no pathological effects, and very slight or negative absorption of aluminium.

Slight positive evidence was adduced by Daniels and Hutton [1925], who reported that rats fed on milk did not reproduce for more than two generations, but that additions of soya bean ash or the equivalent inorganic salts (aluminium, manganese, silicon and fluorine) caused satisfactory reproduction for six generations. The effect of the separate elements was not tested; but Orent and McCollum [1931] have stated that manganese is essential in reproduction.

Toxic effects due to aluminium have been reported by Schaffer *et al.* [1929] working with mice, particularly as to impairment of reproduction, histological examination of the ovary showing that this was markedly affected.

The adequacy of the diet and excessive extent of the aluminium dosage has been questioned by McCollum *et al.* [1929; see also reply by Schaffer *et al.*, 1929]. Bertrand and Serbescu [1931] have reported that the toxicity of aluminium salts injected parenterally is less than that of nickel or copper in the case of rats and rabbits.

Experimental.

It was originally intended to test the effect of raising several generations of rats on a diet entirely devoid of aluminium, as well as to test the effect of adding various levels of aluminium salts to the diet. Unless the former procedure is carried out, effects due to traces of aluminium cannot be excluded. A synthetic ration was employed, each component of which could be purified and tested separately, but certain of these—the vitamin-containing materials and particularly yeast—offered great difficulty. It is almost impossible to eliminate the inorganic constituents from yeast or marmite, yet to prepare vitamin B concentrates, especially at the time when these experiments began, was considered to be beyond the scope of this work. Hence the problem became one of limits—a variety of suitable constituents being tested for aluminium, and those containing the minimum quantity selected for use. Samples of these materials were ashed and examined spectrographically, and Table I gives the maximum and minimum amounts found in each constituent used over a period of 18 months.

Table I. *Maximum and minimum amounts of aluminium found in foodstuffs.*

Material		Starch	Caseinogen	Yeast	Butter	Food mixture
Aluminium in parts per million	Maximum	0·3	0·2	3·7	1·5	0·5
	Minimum	0·1	—	1·2	—	—
Lowest limit observable*		0·1	0·05	0·6	0·1	0·4

* About 1 part of aluminium in 20,000 parts of inorganic ash can be detected, so that the ash content of each substance is the limiting factor in the spectrographic determination of aluminium. The method can be made roughly quantitative [see McCollum *et al.*, 1928].

As the food mixture contained only small portions of yeast (see Table II of rations for details) the limits of aluminium content could be much higher than with the other constituents without unduly raising the total aluminium. Samples of the control ration, therefore, never contained more than 1 part of aluminium in 2 millions. Effects of amounts smaller than this, if such exist, are excluded from consideration here.

The upper limit of aluminium for experiments where this formed a part of the diet was fixed at about 0·08 %. This amount may be administered in the food in the form of phosphate (baking powder residues) or sulphate (alums) without producing cathartic effects, and is analogous to the amount ingested by the human being when living upon a mixed diet [Smith, 1928].

Exp. 1. Thirty rats weighing 50–100 g. were used, 20 receiving the synthetic diet only, the other 10 with the addition of 0·1 % aluminium as soda

alum. (The rations are set out in Table II.) They were allowed as much food as they could consume, together with water. The animals were at first placed in separate glass cages (large battery jars), with wire mesh mats covering the bottom, under which was placed a pad of several layers of filter-paper, the mouth being fitted with a glass plate with gaps for ventilation. Subsequently, the rats were placed in groups of three in the same cages, as they were unfavourably affected by isolation. In later experiments, the use of glass cages was given up, since, although they afforded an environment free from aluminium, this precaution was unnecessary owing to the presence of aluminium in the food, and in general working, glass cages were found to be fragile in use, and extravagant in space and labour. They were only used for special purposes subsequently (*e.g.* metabolic experiments, as described elsewhere [Mackenzie, 1931]).

Table II. *Diets.*

(All components expressed as percentages.)

Diet	Yeast	Caseinogen	Cellulose	Butter	Lard radiostol	Starch	Salt mixture 185	Aluminium* Compound baking powder	Aluminium* Meta
(*a*) Complete:									
A	5	18	2	28	—	43	4	—	—
B	5	18	2	—	—	41	—	2·0†	0·094
C	5	18	2	—	—	39·6	—	3·4	0·1065
(*b*) Vitamin A deficient (?) or absent:									
D	6	22·5	2·5	10	—	54	5	—	—
E	6	22·5	2·5	10	—	51	5	3·0	0·094
F	6	22·5	2·5	—	10	54	5	—	—
G	6	22·5	2·5	—	10	51	5	3·0	0·089
H	6	22·5	2·5	10	—	47·2	5	6·8	0·173
(*c*) Vitamin B deficient (?) or absent:									
V	2	22·5	2·5	20	—	55	—	—	—
W	2	22·5	2·5	20	—	52	—	3·0	0·082
X	0	22·5	2·5	20	—	55	—	—	—
Y	0	22·5	2·5	20	—	52	—	3·0	0·082
Z 1	0	22·5	2·5	—	20	55	—	—	—
Z 2	0	22·5	2·5	—	20	52	—	3·0	0·087

* Actual aluminium content of the food as determined by analysis of samples: not necessarily corresponding to the amount of baking powder residue, which had varying composition and moisture content.

† As soda alum.

The growth curves for rats of Exp. 1 are shown graphically in Fig. 1. The method adopted is to show the average growth for the groups of one sex together with the individual highest and lowest growth curve. It is considered that this gives the fairest computation of the results obtained. Only the growth curves of females are shown in this case, the males showing a general correspondence but about 20 % greater individual weight.

When maximum weight had been reached, and breeding tests were satisfactory, as shown by the production of viable young by each female (see

Table III, under appropriate experiment) a proportion of the animals were killed and the organs analysed for aluminium, using the colorimetric methods of Myers and Morrison [1928]. Owing to the small amount of material obtained from individual rats, it was necessary to carry out these analyses on groups of five or more animals. The results are shown in Table IV.

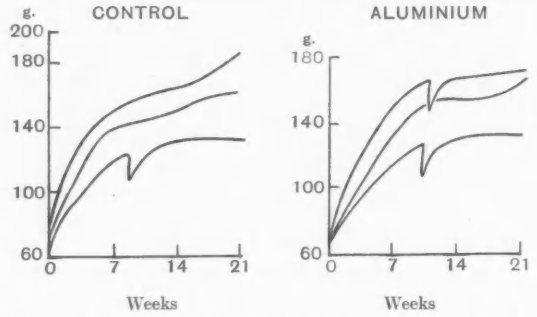

Fig. 1, Exp. 1. Upper curve—heaviest female; lower curve—lightest female; middle curve—average for all females in group.

Table III. *Fertility of animals used in experiments.*

Note. The animals were placed in groups of three, one male and two females as far as possible. If no progeny resulted, the male was considered sterile and a male from another group employed. If only one female produced young, the other was tested with another male before being pronounced sterile. The production of viable young was thus the criterion. Excess males were tested when circumstances allowed.

		Control group				Aluminium group			
Exp.	Sex	Total number	Fertile	Sterile	Not tested*	Total number	Fertile	Sterile	Not tested*
1	♂	4	4	0	—	3	3	0	—
	♀	16	12	0	4	7	5	0	2
2	♂	7	6	1	—	8	5	2	1
	♀	8	6	0	2	7	7	0	—
1 A (first generation)	♂	7	5	0	2	8	5	0	3
	♀	13	10	1	2	10	9	1	—
1 A (second generation	♂	6	4	0	2	8	5	0	3
	♀	9	9	0	—	17	10	0	7
1 A (third generation)	♂	7	6	0	1	11	6	0	5
	♀	12	8	0	4	9	9	0	—

* Including premature deaths.

Exp. 2. This was a repetition of Exp. 1, but the rats were placed in groups of three in glass cages, and the aluminium was supplied in the form of a decomposed baking powder, containing about 3 % of aluminium. To decompose it, this was heated on a water-bath with half its weight of water, and dried at 100°. The product was added to the ration at a level of 3–3·5 %, replacing an equal weight of starch, but as 50 % of the baking powder itself consists of starch, the variation in this item in the diet was insignificant (see ration C in Table II). The experiment lasted 10 months, and the growth

curves, breeding results and analyses appear in the respective Tables and Figures.

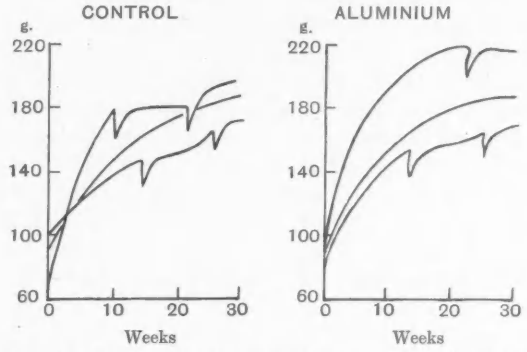

Fig. 2, Exp. 2. Upper curve—heaviest female; lower curve—lightest female; middle curve—average for all females in group.

Exp. 1A et seq. *Effect of aluminium upon subsequent generations.* Progeny of rats employed in Exp. 1, both control and aluminium-fed animals, were

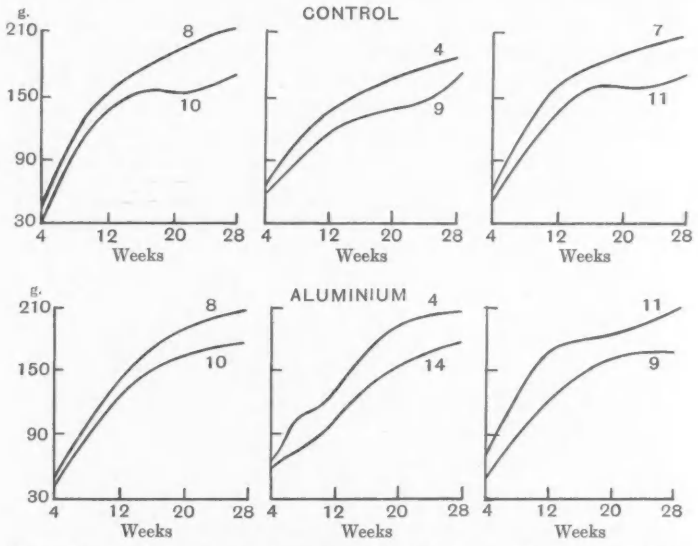

Fig. 3, Exp. 1A. Upper curve—average for all males in group, and number of males. Lower curve—average for all females in group, and number.

used to determine if aluminium at a high or low level could exercise any depressant effect upon growth or fecundity in subsequent generations. The rations used were diets A and C, and three generations were successfully raised. In each generation, about twenty of the young were selected from all

the litters available, and the remainder discarded. In order to shorten the time covered by these experiments, breeding was allowed to commence at the earliest possible age, the sexes not being separated, and the first litters were used as representatives of the next generation. This may explain why the growth curves in all generations are on the low side in both groups. Considerable mortality occurred in the second generation owing to extensive and unavoidable changes of temperature in the cage room during the progress of this experiment. In the case of the aluminised groups, five died, and others from the same generation and on the same diet, which had been set aside for metabolic experiments, were brought in to replace them. In these experiments the rats were placed in the usual type of metal cages in groups of five, the diet being supplied *ad lib.* and unlimited breeding being allowed. The results are shown in growth curves, breeding Table and analysis.

As no perceptible effect due to aluminium could be observed when the diet was sufficient for normal requirements, it was decided to make tests under less favourable conditions, *viz.* when the vitamin factors in the diet were reduced to the lowest level sufficient for maintenance, or below. It seemed possible that any differential effect due to aluminium would be more marked under these conditions.

Exp. 3A. Twenty rats were divided into two groups receiving rations D (control) and E (aluminised) respectively. The rats weighed 40–60 g. at the commencement of the experiment. The fat content and vitamin A, supplied

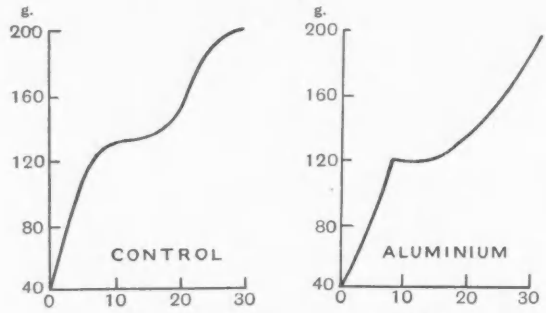

Fig. 4, Exp. 3A. Low vitamin A (males).

in the form of butter, were thus reduced to one quarter of the normal. Growth did not vary significantly between the groups and was little below normal generally, though the mortality was higher. After 11 months, 7 rats remained in the control groups and 5 in the aluminium group, they were killed and used for analysis. Growth curves and analytical results are shown in Fig. 4 and Table IV.

Exp. 3B. *Effect of avitaminosis-A.* Twenty rats, 10 receiving diet F (control) and 10 receiving diet G (aluminised), initial weight about 100 g.,

were employed. The ration contained lard instead of butter, thus excluding vitamin A. The growth curves fell off sharply after 6 or 8 weeks, and no animal survived 4 months after the experiment began, yet no convincing evidence was obtained that aluminium had any accelerating or retarding effect upon the morbidity. In each group individual growth curves of five individuals, including the extremes (earliest and latest deaths) are shown (Fig. 5).

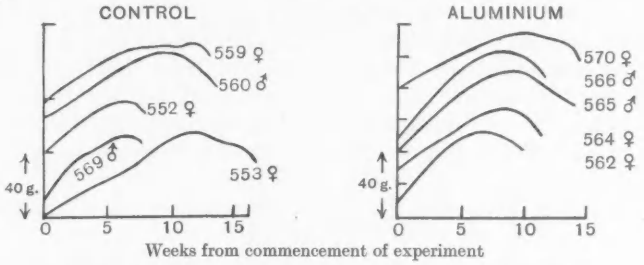

Fig. 5, Exp. 3 B. No vitamin A (animals weighed 100 g. to commence).

Exp. 3 C. *Effect of feeding high levels of aluminium.* Ten rats, being surplus offspring of rats of Exp. 1, were placed on diet G (containing 1·7 % of aluminium as baking powder residue, *i.e.* aluminium phosphate), and their growth was compared with that of 5 rats placed on diet C, similar in all respects except for the omission of the baking powder. Growth was distinctly below normal, but it was noticeable that the rats did not eat the diet readily, and the consumption per head was much less than in the control group (about 9 g. against 13 g.). The ration had a pronounced saline flavour and apparently induced thirst. Analysis of the animals after 6 months on the diet showed no noticeable storage of aluminium in the organs.

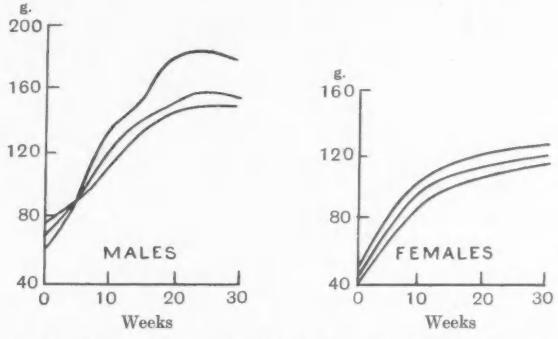

Fig. 6, Exp. 3 C. High aluminium diet (highest, average and lowest growths in group).

Exp. 3 D. *Effect of limiting the vitamin B complex.* A group of 40 rats, about 40–60 g. in weight, was divided into 4 groups of 10, receiving rations

V, W, X and Y (which contained little or no yeast, with and without additions of aluminium). They were caged in groups of five, and coprophagy was prevented as usual. A number of the animals exhibited spasmodic inco-ordination in the early stages, and this was treated by the administration of 10–20 cc. of milk to the affected animal. This produced rapid recovery without supplying excessive amounts of vitamin B. Very few of the animals died during the first 2 months, and the growth curves showed steady if small increases—indicating that butter is a sufficiently rich source of vitamin B when fed at a level of 18 % to maintain growth in the early stages of life. By the sixth month extensive mortality commenced in the control group, while the aluminium group was very little affected. Under these conditions the baking powder addition may be suspected of some specific sparing action or contributory vitamin. The point was not entirely solved by the next experiment.

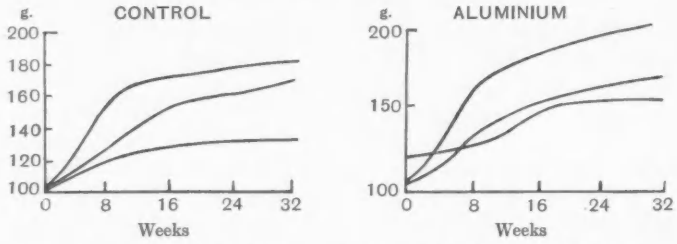

Fig. 7, Exp. 3D. Showing highest, mean and lowest growth curves of the groups.

Exp. 3E. *Effect of aluminium on avitaminosis-B.* Twenty rats were divided into 2 groups of 10, receiving diets similar to X and Y, where lard replaced the butter. Radiostol was also added. The animals were given milk in the early stages whenever inco-ordination was observed, thus effecting a temporary cure, and the experiment was on parallel lines with Exp. 3D. The mortality, as would be expected on a diet entirely deficient in vitamins A and B,

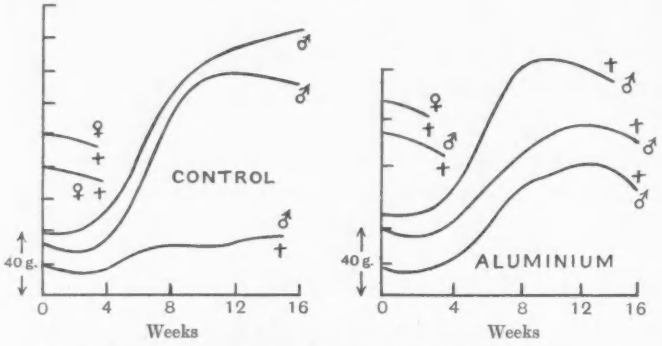

Fig. 8, Exp. 3E. Average weight of animals—50 g. at start; † = death.

was high. In 2 months 7 animals died. The milk supplement was then discontinued, but 8 animals survived after 3 months, and were apparently resistant to avitaminosis-B. After 4 months typical symptoms of avitaminosis-A set in, and only 2 animals (both controls) survived to the fifth month. These rats had shown unusual growth and vigour throughout, and apart from the exceptions, the 2 groups showed no significant difference [see growth curves of Fig. 8, Exp. 3 E].

Discussion.

It is probable that the environment was responsible for the fact that the growth curves in Exps. 1 and 2 were not up to normal, but they showed little difference as between groups on control and aluminised diets. The fertility of rats in these experiments and in the subsequent generations raised from rats of Exp. 1 also show no differential effect. Exp. 3 B, in which high levels of aluminium were fed and some stunting of growth resulted, has some resemblance to the effects obtained by Schaffer *et al.* [1928]. In this case it was notable that the difference in the amount of food consumed would account for the poor growth, the rats taking very little more of it than would suffice for maintenance. To obtain clear-cut results it would be desirable (*a*) to feed a control group with the control diet at the same level as that at which the experimental rats would consume their ration, and compare the growth curves; (*b*) to feed a form of aluminium which is tasteless and non-cathartic, pure aluminium phosphate would probably be suitable, so that the diet would be more freely consumed.

Under present circumstances, the question as to whether the stunting effect is due to a specific action of aluminium, or a general lack of balance in the diet, must be considered an open one. Exp. 3 D, in which an apparent lengthening of life resulted from the aluminium addition, may be considered to be due to a specific action of aluminium, but the other components of the baking powder—starch and albumin—must not be overlooked as a possible source of vitamin B. On the whole, the avitaminosis experiments give no reason to suppose that aluminium has any action one way or the other.

The analytical results suggest that some absorption of aluminium by the liver occurs on aluminised diets. The amounts do not seem to increase cumulatively with the period of feeding, and are quite comparable with the amount found in control animals [see also Tourtellotte and Rask, 1931].

Conclusions.

(1) Aluminium additions to the diet have no perceptible influence
  (i) when the diet is adequate for growth and reproduction in the rat;
  (ii) when the diet is insufficient for growth or maintenance;
  (iii) when fed to several successive generations of rats.

(2) Aluminium absorption from such diets appears to be exceedingly small.

IV. THE OCCURRENCE OF ALUMINIUM IN THE THYROID.

It has been reported by Underhill *et al.* [1929] that aluminium tends to accumulate in the thyroids of dogs when the animals are on an aluminium diet, amounts up to 0·07 mg. having been found in a single thyroid. Recently Tourtellotte and Rask [1931] have questioned these results. Since 1929, as occasions have offered, samples of thyroid have been secured from normal, aluminium-fed and iodine-fed animals in this Institute, in an endeavour to determine whether aluminium is present in the thyroid, and if it can be connected in any way with iodine metabolism. Iodine and aluminium have been determined in each sample (except in two cases where the total amount of tissue would not suffice for aluminium assay, in which case iodine alone was determined). The aluminium content was determined by the colorimetric method using aurintricarboxylic acid [Myers and Morrison, 1928]; the iodine by the method of Leitch and Henderson [1926]. I desire to thank Miss Stuart of this Institute for the iodine determinations referred to below.

(*a*) *Iodine and aluminium in the thyroids of pigs employed for aluminium experiments* [see Mackenzie, 1931].

Pig No.	Diet	Weight of thyroid g.	Iodine in thyroid g./100 g.	Aluminium in thyroid mg./100 g.
2474	Al less than 2 parts per million	4·81	0·155	0·04
2477		4·11	0·165	0·05
2480		2·6 (part)	0·189	0·9
2475	Above diet *plus* 0·1 % aluminium	5·48	0·193	2·63*
2479		4·51	0·202	—†
2488		8·39	0·241	0·02

* Final product showed iodine coloration—result doubtful.
† Results below 0·02 are not reliable, and are left blank.

(*b*) *Iodine and aluminium in the thyroids of pigs employed for iodine experiments.*

Groups of 5 pigs received (I) control ration adequate for normal growth, (II) same diet + potassium iodide, (III) same diet with ultra-violet irradiation, (IV) same diet, with potassium iodide and ultra-violet irradiation.

Group of 5 pigs receiving	Total weight thyroids g.	Iodine in thyroids g./100 g.	Aluminium in thyroid mg./100 g.
Normal diet	18·2	0·118	0·02
,, +KI	29·4	0·238	—
,, +U.V.I.	22·7	0·202	0·06
,, +KI+U.V.I.	34·1	0·097	0·03

Note. The thyroid material had been dissolved in dilute alcoholic potassium hydroxide for iodine determination, and stored in glass bottles. It was anticipated that aluminium might be present owing to the action of the potash on glass, which usually contains aluminium. But the results show that the aluminium content, from whatever source derived, is extremely small.

(*c*) *Other samples of thyroid from pigs.*

Pig	Weight of thyroid g.	Aluminium mg./100 g.
Normal, 6 months old	3·8	0·12
Depancreatised 2 months previously ...	2·1	0·07
Depancreatised 1 day previously ...	4·7	0·05

(*d*) *Iodine in thyroids of rats receiving aluminised diets.*

The thyroid in the rat is too small to be analysed for aluminium by any of the methods now in use. It was considered advisable to find the iodine content of some thyroids in the case of rats used for aluminium experiments, as described above, to see if any correlation might exist. The thyroids of rats in Exps. 1 and 2 were analysed by Miss Stuart, and the results follow:

Exp. No.	No. of rats examined	Average weight of thyroid g.	Iodine content of thyroid g./100 g.
1 (control aluminised)	10	0·017	0·042
	5	0·015	0·061
2 (control aluminised)	6	0·016	0·027
	7	0·019	0·026

These results indicate that

(i) aluminium occurs at only a very low level in the thyroid;

(ii) there is no correlation between aluminium and iodine in normal metabolism.

V. INTESTINAL ABSORPTION OF ALUMINIUM IN THE RABBIT.

The presence of small quantities of aluminium in the normal diet of men and animals [Smith, 1928], and the presence of aluminium in very minute quantities in the tissues [Myers and Mull, 1928], are both well established facts which have not been satisfactorily correlated. It is established that certain aluminium compounds, when present in the food, are converted into a soluble form by the action of the gastric juice [Myers and Killian, 1928] and it seems reasonable to suppose that in the upper part, at least, of the small intestine, absorption of the aluminium ions may be expected to occur as is the case with calcium, sodium and potassium, though in the lower parts of the intestine aluminium appears to be precipitated [Massatch, 1930]. The absorbed aluminium might be conveyed by the portal blood to the various organs, notably to the liver, and it has been observed that the aluminium content of the liver is greater in aluminium-fed animals than in controls. Though the amounts are in all cases very small [Underhill *et al.*, 1929] the increase is definite, and can hardly be explained on any other ground. But the presence of detectable amounts of aluminium in the blood after ingestion of aluminium compounds has rarely been observed, the findings of all workers being negative except those of Underhill *et al.* [1929]. These workers, using an exceptionally sensitive method for the detection of aluminium, found that aluminium was sometimes

absent from the blood of normal animals, and even when present the initial amount was not always increased by aluminium ingestion, while in some cases a fall was observed.

Myers and Morrison [1928] injected a known amount of an aluminium compound, usually the sulphate or chloride, into a washed intestinal loop in dogs, and found it possible to recover the aluminium, within the limits of experimental error, after a period of 1 to 4 hours. They pointed out, however, that the aluminium was recovered as a precipitate on the mucosa, and it seems certain that the normal reaction of the intestine would convert most aluminium salts to the hydroxide, which is colloidal and probably non-absorbable.

In the present experiments, a known amount of aluminium in the form of a solution of aluminium tartrate was injected into the ligatured intestine of a rabbit (which had received an aluminium-free diet for one week previous, and had fasted for 24 hours before the experiment) by the method due to Magee and Macleod [1928]. The rabbit, anaesthetised with urethane and ether, was opened up to expose the viscera, and the intestine tied off at the oesophagus, the ileal extremity being cut and sutured to a discharge tube. A small cannula was inserted below the ligature, and 10 cc. of aluminium tartrate solution (1 mg. Al per cc.) at approximately p_H 8 were injected. After an interval varying from 15 to 30 minutes, about 20 cc. of blood were drawn from the portal vein by hypodermic syringe; a second sample was drawn after a further period of 10 to 15 minutes. The animal was then bled to death through a cannula in the carotid artery, and subsequently the liver and entire small intestine were dissected out. These samples of blood and tissue were ashed in silica basins and the aluminium content was determined by the colorimetric method. In

Table IV. *Aluminium found in the blood of rabbits after injection of aluminium tartrate into intestine.*

								Control	
Exp. No. ...	1	2	3	4	5	6	7	8	9
Aluminium injected into intestine (mg.)	11·05	10·8	10·4	11·05	11·05	10·3	11·05	0·0	0·0
Aluminium found (mg./100 g.):									
(*a*) in portal blood									
1st sample	0·017 (20)	0·047 (30)	0·000 (15)	0·016 (15)	0·000 (15)	0·000 (25)	0·000 (25)	0·000	0·016
2nd sample	0·049 (35)	0·000 (45)	0·021 (45)	*	0·000 (25)	0·000 (35)	0·000 (40)	—	—
(*b*) in systemic blood	0·000 (40)	0·000 (50)	—†	—	5·60‡	0·000 (40)	0·083 (45)	0·000	0·000
(*c*) in the liver	0·56	0·86	—	0·42	0·79	0·55	1·15	1·12	0·70
Aluminium found in intestine (mg.)	1·48	8·82	9·04	11·3	9·6	7·73	9·6	0·67	0·57
Percentage recovered from intestine	13·5	81·7	86·9	102·2	87·7	75·1	87·7	—	—

Average percentage recovered in six experiments ... ... 86·8

The figures in brackets following the blood figures are the times (mins.) from the initial injection at which the blood sample was taken.

* This animal died 25 minutes from commencement.
† This animal died 45 minutes from commencement.
‡ This animal died 30 minutes from commencement. The systemic blood sample was obtained from the body cavity, and was possibly contaminated by the liquid injected into the intestine—the actual amount of aluminium present in 10·7 g. of blood was 0·60 mg.

addition to 7 animals thus used, 2 others were used as controls, 10 cc. of plain saline being injected into the intestine, the other operations being as before. The results are shown in Table IV.

Discussion.

The average recovery in 6 experiments varied from 75·1 % to 102·2 % with a mean value of 86·8 %. The first experiment showed an extremely low recovery of aluminium from the intestine, possibly due to leakage at the site of injection. It stands alone and has been ignored in computing the average. In the 2 control animals, the aluminium level in the intestine was about 5 % of that found in the experimental animals, showing that 7 days on an aluminium-free diet was not sufficient to remove all traces of aluminium from the intestine. Hence it appears probable that the average recovery of aluminium injected is only about 82 %.

If the aluminium content of the portal blood is compared with the amount present in the intestine, it does not seem probable that aluminium is readily absorbed by the intestine. The amount circulating in the blood at any time is extremely small, but it is undeniable that aluminium may be, and frequently is, conveyed in the blood-stream.

Summary.

Injection of aluminium salts into the intestine of rabbits does not cause any appreciable absorption of aluminium into the blood-stream. The aluminium injected can be largely recovered by analysis of the intestine.

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CIII. EFFECT OF ASSOCIATED GROWTH ON FORMS OF LACTIC ACID PRODUCED BY CERTAIN BACTERIA[1].

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Specificity in the production of a given form of lactic acid by cells functioning under normal conditions is usually assumed to be a fixed character. While this may be true, such cells are not lacking in a mechanism for the production of both enantiomorphs. Thus, for example, while the animal cell *in vivo* seems to produce *d*-lactic acid exclusively, Embden and his associates [1912; Oppenheimer, S., 1912; Loeb, 1913; Oppenheimer, M., 1914] found that the enzyme preparations of ground liver, muscle, *etc.* produced both forms, and in some cases produced an excess of *l*-lactic acid. An even more pronounced reversal is reported by Neuberg [1913] in the production of pure *l*-lactic acid from methylglyoxal by the action of muscle and liver extracts.

In the field of microbiology many apparently inconsistent results have been reported. Some investigators [Pottevin, 1898; Péré, 1893, 1898; Kantardjieff and Pappow, 1931; Orla-Jensen, 1919] have asserted that the form of lactic acid varied with the type of carbohydrate, the form of nitrogen, and the temperature of incubation. Others [Kozai, 1899; Nencki, 1891; Currie, 1911–12] have been unable to bring about any such changes in the form of lactic acid by varying the conditions of fermentation. It is impossible to harmonise these conflicting reports but it is probable that some of the contradictions are due to impure cultures and others to faulty analytical procedures such as fractionation of the zinc salts and incorrect interpretation of the data on specific rotation. Most of the papers which report a modification in the form of lactic acid deal with a change from the production of both enantiomorphs to that of but one. Perhaps the most plausible explanation for this change is that of Orla-Jensen [1919]. He concludes that two enzymes, one producing the dextro-acid, the other the laevo-acid, are present in the cell but that under definitely unfavourable conditions the activity of one of the enzymes is inhibited and hence an active acid results. Further support of

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this view is found in the fact that while newly isolated cultures may produce both forms, the same cultures after repeated transfers in the laboratory may lose their ability to produce one of the enantiomorphs.

In the formation of lactic acid from methylglyoxal, using unaltered washed cells, Neuberg and Gorr [1925, 1, 2, 1926], Neuberg and Kobel [1927], Neuberg and Simon [1927, 1931] found an active lactic acid in two cases. However, using enzyme preparations, inactive acid was obtained in all cases. Apart from the possible influence of the substrate, methylglyoxal, on the form of acid produced, the treatment of the cells employed seems to be of primary importance. Where intact resting cells are used there seems to be a possibility of active acid production, but with enzyme preparations inactive acid is formed.

From these facts it appears probable that all cells contain enzymes capable of producing both forms of lactic acid, but that under certain conditions the activity of these enzymes is so controlled that only one enantiomorph appears.

In a former paper [1926] it was reported that a modification of the form of lactic acid could be obtained without disturbing normal growth and reproduction of the cells. This change was accomplished by growing the lactic acid organism in association with a non-lactic acid-producing microorganism. In a mixed culture of *L. leichmanni* and *Cl. acetobutylicum* inactive lactic acid was produced. By itself the former produces only *l*-lactic acid and the latter, in so far as it has been possible to determine, produces no lactic acid at all. Lactic acid-producing bacteria (*L. leichmanni*, and related forms) are frequently found as contaminants in the industrial production of acetone and butanol by *Cl. acetobutylicum*. The association of these two microorganisms is therefore a more or less natural one. The validity of our report has been questioned by Miss Stephenson [1930], who says, "Such a phenomenon would, however, need very rigorous proof before it could be established." We have repeated the work and extended it to various other combinations of microorganisms and find no errors in the former results.

Cl. acetobutylicum affects dextro- and laevo-lactic acid-producing organisms in the same way, *viz.* by causing them to form inactive lactic acid. This property seems to be peculiar to *Cl. acetobutylicum* and is not possessed by closely related butyric acid-forming bacteria. Likewise yeasts, which are usually grown in association with lactic acid-producing organisms, have no influence on the form of acid produced by the latter.

Experimental.

Cultures and media. Pure cultures of the following organisms were used: *Lactobacillus leichmanni*, strains A and D; *Lactobacillus delbrücki*, strains 3 and 4; *Lactobacillus casei*; *Lactobacillus bulgaricus*, strain 18; *Lactobacillus acidophilus*; *Streptococcus lactis*, strain R; *Leuconostoc pleofructi*, strain 3; *Saccharomyces cerevisiae*, strains RS, and 53; *Saccharomyces ellipsoideus*, strain E; *Clostridium acetobutylicum*, strains 105, 300 and 500; *Clostridium* sp., strain 21; and *Aerobacillus polymyxa* (Prazmowski), strain 35.

In testing the yeast cultures alone for the production of lactic acid a 20 % glucose medium was used. A solution containing 3 % glucose and 3 % suspension of malt sprouts was used as a culture medium in all other cases. In experiments dealing with the associated growth of two organisms, both organisms were inoculated simultaneously, except in one case where *Cl. acetobutylicum* was grown a short time before inoculating with *L. casei*. After 24 hours' incubation at 37° an excess of sterile calcium carbonate was added. The degrees of growth of the lactic acid-producer and of *Cl. acetobutylicum* were indicated by the production of non-volatile and volatile acid respectively.

Microscopic examinations of the mixed cultures were made from time to time, the results substantiating the conclusions drawn from the volatile and non-volatile acid-production.

Analytical methods. Sugar, volatile acid and non-volatile acid were determined essentially as described in previous publications. However, instead of ethyl ether, *iso*propyl ether was used for the extraction of the non-volatile acid. *iso*Propyl ether gives a solution that is freer from coloured impurities and yields purer zinc salts than does ethyl ether. Zinc lactate was prepared by boiling the extracted lactic acid with an excess of alkali-free zinc carbonate, decolorising and crystallising from 50 % alcohol. In all cases 75 % or more of the acid was obtained as the zinc salt. In all cases an effort was made to obtain as much of the salt as possible. The salt was dried over $CaCl_2$ and the water of crystallisation determined by heating the salt at 105° for 4 hours. The water of crystallisation of the pure inactive salt is 18·12 % while that of the active salt is 12·89 %. The optical rotation of the salt was determined for a 4 % solution in a 2-dm. tube. The rotation of the salt is opposite to that of the free acid.

Form of lactic acid in pure culture. Table I shows the form of lactic acid produced by nine cultures. Four of these were found to produce *l*-lactic

Table I. *From of lactic acid produced by pure cultures.*

Organisms	Glucose fermented % of total	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic g. per 100 cc.	Water of crystallisation of zinc lactate %	Specific rotation $[\alpha]_D^{20}$	Form of lactic acid
L. leichmanni, A	90	2·7	0·08	2·64	13·23	+8·22	Laevo
L. leichmanni, D	87	2·5	0·04	2·37	13·03	+8·65	Laevo
L. delbrücki, 4	97	2·9	0·09	2·2	13·03	+8·00	Laevo
Leu. pleofructi, 3	97	2·9	0·09	0·64	13·15	+8·10	Laevo
L. delbrücki, 3	87	2·8	0·06	2·32	13·32	−7·36	Dextro
L. casei	83	3·0	0·03	2·8	13·26	−8·65	Dextro
S. lactis, R	90	2·6	0·01	2·41	13·23	−8·65	Dextro
L. bulgaricus, 18	93	2·8	0·06	1·81	15·50	—	Mixture
L. acidophilus	98	2·1	0·08	2·11	18·08	0·0	Inactive

acid, *viz. L. leichmanni*, strains A and D; *Leu. pleofructi*, 3; and *L. delbrücki*, strain 4. Three were found to produce the *d*-acid, *viz. L. casei*; *L. delbrücki*,

strain 3; *S. lactis*. The others, *L. acidophilus*, and *L. bulgaricus* were found to produce mixtures of *d*- and *l*-lactic acid.

Table II gives the results of experiments with various non-lactic acid-producers in order to make certain that no lactic acid was formed. No test for

Table II. *Test of yeasts and butyric acid-forming organisms for the production of lactic acid.*

Organisms	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic* g. per 100 cc.
S. cerevisiae, RS	19·2	0·08	0·15
S. cerevisiae, 53	19·2	0·08	0·15
S. ellipsoides, E	19·2	0·08	0·20
Cl. acetobutylicum, 500	2·9	0·87	Trace
Cl. sp., 21	2·9	0·53	Trace
A. polymyxa, 35	2·8	1·84	Trace

* In all cases the thiophen test for lactic acid was negative.

lactic acid with thiophen was obtained from the non-volatile extracts of any of these fermentations, nor was it possible to obtain any zinc lactate in these cases. The volatile acid production was determined as an index of growth of these organisms.

Table III. *Effect of association on the form of acid produced by l-lactic acid organisms.*

Organisms	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic g. per 100 cc.	Water of crystallisation of zinc lactate %	Form of lactic acid
L. leichmanni, A and *Cl. acetobutylicum*, 105	2·8	0·39	1·71	18·16	Inactive
L. leichmanni, A and *Cl. acetobutylicum*, 105	2·9	0·42	1·66	18·16	Inactive
L. leichmanni, A and *Cl. acetobutylicum*, 105	2·9	0·44	1·58	18·07	Inactive
L. leichmanni, A and *Cl. acetobutylicum*, 500	2·9	0·11	1·02	18·20	Inactive
L. leichmanni, D and *Cl. acetobutylicum*, 105	2·6	0·45	0·48	18·03	Inactive
L. leichmanni, D and *Cl. acetobutylicum*, 500	2·6	0·31	0·31	18·03	Inactive
L. delbrücki, 4 and *Cl. acetobutylicum*, 500	2·8	0·17	1·38	18·00	Inactive
L. delbrücki, 4 and *Cl. acetobutylicum*, 500	2·9	0·15	1·42	18·04	Inactive
L. delbrücki, 4 and *Cl. acetobutylicum*, 500	2·8	0·23	1·70	18·18	Inactive
Leu. pleofructi, 3 and *Cl. acetobutylicum*, 500	2·9	0·79	0·37	17·50	Inactive
Leu. pleofructi, 3 and *Cl. acetobutylicum*, 500	2·9	0·57	0·37	18·20	Inactive
Leu. pleofructi, 3 and *Cl. acetobutylicum*, 500	2·9	0·73	0·34	17·78	Inactive
Leu. pleofructi, 3 and *Cl. acetobutylicum*, 500	2·9	0·75	0·15	17·65	Inactive
L. leichmanni, A and *S. cerevisiae*, 53	2·9	0·08	1·89	13·31*	Laevo
L. leichmanni, A and *Cl. acetobutylicum*, 105†	2·9	—	1·50	12·97*	Laevo
L. leichmanni, A and *Cl. sp.*, 21	2·34	0·12	2·07	13·14*	Laevo
L. leichmanni, A and *A. polymyxa*, 35	2·31	0·12	1·75	13·32*	Laevo

* Specific rotation of these samples varied from +7·8° to +8·3°.

† Culture 105 was grown for 24 hours in the medium which was then sterilised and inoculated with culture A. Volatile acid was not determined because the medium was to be sterilised and re-inoculated.

Form of lactic acid produced in mixed cultures. As shown by Table III all the *l*-acid-formers, when grown with *Cl. acetobutylicum*, produced *i*-lactic acid, *i.e.* a mixture of *d*- and *l*-acids. When grown with other non-lactic acid-producers, yeasts and butyric acid bacteria, no change of form was detected

although the volatile acid production of the butyric acid bacteria indicated a satisfactory growth. An important point in the association with *Cl. acetobutylicum* is that there is obtained an equimolecular proportion of *d*- and *l*-acids, as indicated both by the water of crystallisation and by the optical inactivity. It should also be observed that among these *l*-acid-producing cultures there are representatives of three distinct classes of organisms.

The effect of associated growth on the *d*-lactic acid-producers is shown by the data in Table IV. The streptococcus, R, easily gave a change of form of

Table IV. *Effect of association on the form of acid produced by* d-*lactic acid organisms.*

Organisms	Glucose fermented % of total	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic g. per 100 cc.	Water of crystallisation of zinc lactate %	Form of lactic acid
Strep. lactis, R and *Cl. acetobutylicum*, 500	96	2·9	—	1·11	16·47	Dextro and inactive
Strep. lactis, R and *Cl. acetobutylicum*, 500	92	2·8	0·70	0·46	14·71	Dextro and inactive
Strep. lactis, R and *Cl. acetobutylicum*, 500	92	2·8	0·44	1·16	18·22	Inactive
Strep. lactis, R and *Cl. acetobutylicum*, 500	92	2·8	0·40	1·11	18·20	Inactive
Strep. lactis, R and *Cl. acetobutylicum*, 500	92	2·8	0·44	0·94	18·23	Inactive
Strep. lactis, R and *S. cerevisiae*, 53	97	3·2	0·06	0·39	12·51	Dextro, specific rotation of zinc salt −8·65°
L. casei and *Cl. acetobutylicum*, 300	91	3·0	0·05	0·81	13·49	Dextro, specific rotation of zinc salt −7·8°
L. delbrücki, 3 and *Cl. acetobutylicum*, 500	91	3·0	0·11	1·77	13·44	Dextro, specific rotation of zinc salt −8·37°
L. delbrücki, 3 and *Cl.* sp., 21	77	2·3	0·04	1·83	12·50	Dextro
L. delbrücki, 3 and *Cl.* sp., 21	97	2·9	0·17	1·53	12·06	Dextro
L. casei and *Cl. acetobutylicum*, 500*	96	2·8	0·40	1·08	16·2	Dextro and inactive
L. casei and *Cl. acetobutylicum*, 500*	96	2·8	0·40	0·54	15·55	Dextro and inactive

* *Cl. acetobutylicum* grown for 24 hours before inoculation with *L. casei*.

lactic acid, this change being from *d*- to *i*-acid. It was early discovered from microscopic examinations and from volatile acid determinations that the *d*-acid-producing bacteria exerted an inhibitory effect on the acetone-butyl alcohol organism. Where the medium was inoculated simultaneously with both organisms no growth of *Cl. acetobutylicum* took place and the lactic acid produced was consequently active. Therefore the associated organism was grown for 24 hours before inoculation with *L. casei*. Apparently in this case

a sufficient number of cells of the associated organism were present and the acid formed was a mixture of *d*- and *i*-acids. It is probable, since the acetone-butyl alcohol organism did not cause appreciable evolution of gas after the addition of *L. casei*, that the former no longer grew actively, and some of the acid produced by the lactic organisms was active, as is that formed when the two organisms are inoculated simultaneously.

Some properties of the active agent. That the shift in the form of acid can be brought about only through the agency of the living cell or some heat-labile substance elaborated by it was demonstrated by us [1926] and confirmed by the following experiment. A 24-hour culture of *Cl. acetobutylicum* was sterilised. This treatment would destroy enzymes and other heat-labile substances but would leave intact the other fermentation products. The medium was then inoculated with *L. leichmanni* and incubated for several days. An abundance of lactic acid was produced but it was all laevorotatory.

Next an effort was made to determine if the agent responsible for the change in the form of lactic acid could pass through a collodion or viscose membrane (Table V). Sacs made of these materials were filled with medium

Table V. *Experiments with viscose and collodion sacs.*

Organisms	Type of sac	Test for*		Water of crystallisation of zinc lactate %	Specific rotation $[\alpha]_D^{20}$	Form of lactic acid	Remarks
		Cl. acetobutylicum in lactic culture	Lactic acid organism in *Cl. acetobutylicum* culture				
L. leichmanni, A and *Cl. acetobutylicum*, 500	Collodion	+	+	18·10	0·0°	Inactive	Organisms passed through membrane
L. leichmanni, A and *Cl. acetobutylicum*, 500	Collodion	+	+	18·20	0·0°	Inactive	Organisms passed through membrane
L. leichmanni, A and *Cl. acetobutylicum*, 500	Collodion	+	+	18·00	0·0°	Inactive	Organisms passed through membrane
L. leichmanni, A and *Cl. acetobutylicum*, 500	Viscose	–	–	13·3	+7·8°	Laevo	No effect of *Cl. acetobutylicum*
L. leichmanni, A and *Cl. acetobutylicum*, 500	Viscose	–	–	13·2	+7·9°	Laevo	No effect of *Cl. acetobutylicum*
L. casei, and *Cl. acetobutylicum*, 500	Viscose	–	–	13·5	−8·65°	Dextro	No effect of *Cl. acetobutylicum*
L. casei, and *Cl. acetobutylicum*, 500	Viscose	–	–	13·5	−8·22°	Dextro	No effect of *Cl. acetobutylicum*

* Growth of *Cl. acetobutylicum* was tested for on the lactic side of the membrane by inoculation into corn-mash. Growth of lactic acid organisms was tested for on the *Cl. acetobutylicum* side of the membrane by streaking on a glucose peptone agar plate.

and were suspended in some more of the same medium. The medium inside the sac was inoculated with one organism and that outside the sac with the other organism. Collodion sacs proved unsatisfactory as the two organisms penetrated the membrane and were found both inside and outside the sac. *i*-Lactic acid was formed as a result of the intimate growth of the two organisms. Viscose sacs were found to keep the two organisms separated throughout the fermentation, but an active lactic acid was formed in such

fermentations. It seems probable that most of the products of *Cl. acetobutylicum* passed through the membrane, since similar viscose sacs have been found to be highly permeable to aniline dyes and plant viruses. It would appear, therefore, that the two types of cells must be in intimate contact or that the influential product of *Cl. acetobutylicum* is incapable of passing through the membrane.

SUMMARY.

The effect of associated growth on the optical form of lactic acid produced by certain bacteria has been studied. Four *l*-lactic acid-forming bacteria, and three *d*-acid-producers were grown with four non-lactic organisms, in various combinations.

When the lactic acid bacteria, which by themselves produce only *d*- or *l*-acid, were grown in association with the acetone-butyl alcohol organism, *Cl. acetobutylicum*, *i*-lactic acid was formed.

Intimate contact between the lactic acid bacteria and *Cl. acetobutylicum* seems to be necessary to bring about the change since no effect was obtained when the two organisms were separated by a viscose membrane. The active agent is apparently not diffusible and is destroyed by heat.

The ability to bring about this change in the form of lactic acid seems to be the peculiar property of *Cl. acetobutylicum*. Closely related butyric acid bacteria had no effect. The yeast, *S. cerevisiae*, did not bring about any change in the form of lactic acid.

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CIV. THE INCEPTION OF BLOOD CLOTTING.

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I. *Introduction.*

Two explanations of the inception of the spontaneous clotting of pure blood are current. One group of investigators suggests that the initial change is the breakage of blood corpuscles and therefore attributes the inauguration of coagulation to a process akin to cytolysis. Of these inquirers, Morawitz [1904] regards the disintegration of both leucocytes and blood platelets as primary occurrences, but Cramer and Pringle [1913] limit the initial change to the lysis of platelets, whilst Fuchs [1930] maintains that, although the products liberated from leucocytes may contribute to the coagulation of slowly clotting blood, the dissolution of platelets is the primary occurrence when coagulation is rapid. The second group of investigators believes that the inception of blood clotting is primarily a humoral reaction in which changes in the blood-plasma precede the breakage of corpuscles and the release of participants in coagulation from these bodies [Bordet, 1920; Pickering and Hewitt, 1921; Mason, 1921; Pickering and de Souza, 1923; Mills and Mathews, 1924; Mills, 1928; Pickering, 1928]. A plasmatic reaction in the inauguration of blood clotting is also implied by those physiologists who accept the teaching of Wooldridge [1893] that blood platelets are artefacts, but the latter conclusion is rejected by almost all those haematologists who have made a special study of the blood platelets [*vide* Roskam, 1927; Cramer and Bannerman, 1929 and the recent review of this subject by Mackay, 1931]. Fundamentally different explanations are also offered for the maintenance of the fluidity of the blood after the introduction into the blood-stream of certain bodies which induce clotting in shed blood. One group of investigators postulates that fluidity is maintained *in vivo* by the hepatic secretion of an excess of an anticoagulant [Morawitz, 1904; Mellanby, 1909; Collingwood and MacMahon, 1912–13; Howell, 1925, 1928; Fuchs, 1930]. But several other workers either wholly or partly reject this suggestion and suggest that the essential difference in the condition of circulating and shed blood is the alteration or disintegration of plasma complexes in the latter fluid [Bordet, 1920; Pickering and Hewitt, 1921; Mason, 1921, 1924; Pickering and de Souza, 1923; Pickering, 1925, 1928; Mills, 1928].

The production of intravascular clotting by the intravenous injection of extracts of various tissues is well known but its relationship with natural thrombosis is still obscure.

A controversy has existed for more than half a century on the mode of action of the participants in blood clotting commonly known as thrombokinase and thrombin in this country. These bodies are described as enzymes by one group of inquirers [Morawitz, 1904; Mellanby, 1909; Waldschmidt-Leitz *et al.*, 1929], but several other investigators claim that they react quantitatively in the coagulation of the blood [Rettger, 1909; Bordet, 1920; Howell, 1925, 1928; Mills and Mathews, 1924; Mills, 1928].

A large proportion of the experiments upon which these divergent conclusions are based were conducted with either blood or blood-plasma to which anticoagulants had been added, and an adequate comparison of the results obtained *in vitro* with those obtainable *in vivo* is often lacking. It seemed advisable to re-investigate some of the principal phenomena of blood clotting during the maintenance of the fluidity of the blood in paraffined vessels, without the addition of an anticoagulant, and to compare the results so obtained with parallel reactions *in vivo*.

II. *General technique.*

Cats, anaesthetised with chloralose, were used. Artificial respiration was only employed in the experiments on intracardiac blood after the isolation of the heart from the circulation by ligatures. Substances introduced into the blood-stream were injected into the jugular vein. A hypodermic syringe was used to inject material into the isolated heart. Shed blood was obtained from the carotid or femoral arteries. A freshly prepared paraffined cannula and a fresh incision into the artery were used for each bleeding. The animals were kept on the operating table for an hour before blood was shed to avoid the fluctuations in the speed of blood clotting which commonly occur shortly after the administration of an anaesthetic. Only relatively small quantities of blood were used in the experiments on shed blood to avoid the hypercoagulability of the blood produced by haemorrhage. It was found that amounts up to 15 cc. of blood could be withdrawn from an animal in good condition without an appreciable variation in the speed of blood clotting. Every experiment on changes induced in the coagulability of blood was accompanied by a control experiment. Except where otherwise stated, all experiments on shed blood were conducted in paraffined vessels.

The blood-serum employed was prepared from blood shed through a paraffined cannula by centrifuging broken-up blood clots directly the coagulation was complete. It was immediately used. The fresh tissue juices were prepared by crushing and grinding the tissues with sand in a mortar, after washing the tissue in normal saline and removal of connective tissue, and the juices were obtained by centrifuging. They were immediately used. The animals' own tissues were employed.

By the expression "commencement of clotting" is implied the first visible departure from fluidity other than the formation of isolated filaments of minute dimensions on the surface of the blood exposed to the air, by "completion," that coagulation had advanced so far that the vessel could be inverted without spilling. The speeds of clotting are given in minutes and seconds. Each experiment is representative of a number yielding concordant results.

III. *The coagulation of blood-plasma after the removal of leucocytes.*

The experiments illustrated in Tables I and II show that the removal of the leucocytes from the blood-plasma in an intact condition does not retard the coagulation of that fluid. It thus appears that the disintegration of leucocytes is not an essential factor in the coagulation of the blood.

The leucocytes were removed from the plasma by centrifuging the blood in paraffined vessels under liquid paraffin at the temperature of the laboratory. Histological examination did not reveal any damage to the leucocytes and plasma was selected in which an abundance of platelets remained intact.

Table I. *Illustrating the speeds of the coagulation of blood-plasma on glass before and after the removal of the leucocytes.*

Temperature 18°.

No. of exp. ...	1	2	3
	Whole blood	Blood-plasma containing leucocytes and platelets	Blood-plasma containing platelets but no leucocytes
Commencement of clotting	6′ 0″	6′ 10″	6′ 0″
Completion of clotting	6′ 40″	6′ 35″	6′ 30″
No. of exp. ...	4	5	6
Commencement of clotting	4′ 0″	3′ 55″	3′ 50″
Completion of clotting	4′ 20″	4′ 10″	4′ 8″

The rapidly clotting blood was obtained from an animal after haemorrhage. 0·5 cc. of blood or plasma was used in each experiment.

Table II. *Illustrating the speeds of the coagulation of blood-plasma in a paraffined vessel before and after the removal of leucocytes.*

Temperature 18°.

No. of exp. ...	7	8	9
	Whole blood	Blood-plasma containing leucocytes and platelets	Blood-plasma containing platelets but no leucocytes
Commencenent of clotting	56′ 0″	57′ 10″	53′ 30″
Completion of clotting	62′ 10″	61′ 20″	60′ 50″

0·5 cc. of blood or plasma was used in each experiment. A repetition of the experiment with a hypercoagulable blood obtained after haemorrhage gave similar results.

IV. *The rôle of blood platelets in the inception of clotting.*

The next group of experiments (Nos. 10–19) shows that the complete disintegration of blood platelets neither produces intravascular clotting nor hastens the coagulation of normal blood which has been shed through a paraffined cannula into paraffined vessels. They also illustrate that the lysis of platelets accelerates the clotting of blood which has been disturbed either by contact with glass (which is wetted with blood) or by the presence of tissue débris. It is concluded that the breakdown of platelets is not the primary occurrence in the clotting of pure blood; but that the products liberated by the disintegration of platelets rapidly induce coagulation in blood-plasma which has been disturbed either by contact with a surface which it wets or by the action of tissue débris.

Exp. 10. 5 cc. of a 10 % solution of saponin in normal saline were injected into the jugular vein of an anaesthetised cat weighing 3½ kg. Blood shed from the carotid artery 10 mins. later showed complete lysis of platelets, indications of damage to some of the leucocytes and partial haemolysis of the erythrocytes. *Post mortem* examination revealed absence of intravascular clotting. The times of coagulation at 18° of blood shed on glass before the injection of saponin were: commencement of clotting 5′ 30″, completion of clotting 6′ 10″; after the administration of saponin: commencement of clotting 4′ 20″, completion of clotting 5′ 10″.

Exp. 11. 0·5 cc. of distilled water was injected into each of the ventricles of the heart of a cat after the isolation of the heart from the general circulation by ligatures. Histological examination of blood aspirated from the ventricles revealed almost complete lysis of platelets. There was no intracardiac clotting.

In each of the experiments summarised in Tables III, IV and V, blood from the carotid artery was shed into two paraffined vessels. The blood in one vessel was mixed with sufficient saponin to disintegrate all the platelets.

Table III. *Illustrating the speeds of clotting in paraffined vessels at 19° of arterial blood and of arterial blood in which the platelets had been disintegrated by saponin (0·04 cc. of a 10 % solution of saponin to 0·5 cc. of blood was used).*

No. of exp. ...	12	13	14	15
	A small drop of pure blood	0·5 cc. of pure blood	A small drop of blood + saponin	0·5 cc. of blood + saponin
Commencement of clotting	57′ 0″	64′ 0″	57′ 30″	64′ 10″
Completion of clotting	60′ 0″	66′ 0″	60′ 30″	68′ 0″

There was no indication of hypercoagulability of this blood since the clotting times on glass at 39° were: commencement of clotting 3′ 30″, completion of clotting 4′ 0″. The same concentration of saponin was used in the experiments noted in Tables IV and V.

Table IV. *Illustrating the speeds of clotting in glass vessels at 19° of arterial blood and of arterial blood in which the platelets had been disintegrated by saponin before the blood had been placed on glass.*

No. of exp. ...	16	17	18	19
	0·5 cc. of pure blood	0·5 cc. of blood + saponin	2·5 cc. of pure blood	2·5 cc. of pure blood + saponin
Commencement of clotting	6′ 15″	4′ 0″	6′ 40″	4′ 0″
Completion of clotting	9′ 5″	4′ 15″	7′ 25″	7′ 10″

Table V. *Illustrating the speeds of clotting in paraffined vessels at the temperature of the laboratory (19°–20°) of extremely hypercoagulable blood, unmixed with saponin and after admixture with saponin.*

(The blood had been rendered hypercoagulable by contact with tissue débris.)

No. of exp. ...	20	21	22
	The blood in a glass vessel	The blood in a paraffined vessel	The blood + saponin in a paraffined vessel
Commencement of clotting	Not determined	16′ 25″	4′ 30″
Completion of clotting	0′ 3″	18′ 25″	10′ 30″

V. *The difference in the capacity of fresh blood-serum to clot shed and unshed blood.*

Exps. Nos. 23 to 26 illustrate that fresh blood-serum promptly clots both pure blood and pure blood-plasma which have been shed into paraffined vessels. Those numbered 27 and 28 show that all the blood in the ventricles of the heart of male cats and of non-pregnant female cats, after isolation of the heart from the circulation by ligatures, remains completely fluid *in vivo* after the injection of fresh serum, although the presence of active thrombin can be demonstrated by the capacity of the intracardiac blood to clot oxalated plasma. A fundamental difference is thus apparent in the resistance of unshed and shed blood to coagulation by the thrombin of serum which cannot be explained as due to the neutralisation of thrombin *in vivo* by an anticoagulant but is consistent with the conclusions of Pickering [1925, 1928] and of Mills [1928] that dissociation of plasma-colloids is a pre-requisite for the coagulation of the blood by thrombin. Exp. No. 29 shows, however, that if the heart is

Table VI. *Illustrating the rapid clotting by fresh serum of pure blood and of pure blood-plasma shed into paraffined vessels.*

Temperature 19°.

No. of exp. ...	23	24	25	26
	5 cc. of blood + 1 % of serum	15 cc. of blood + 0·3 % of serum	2 cc. of plasma + 0·1 % of serum	10 cc. of plasma + 0·2 % of serum
Commencement of clotting	—	0′ 8″	2′ 15″	3′ 0″
Completion of clotting	2′ 30″	8′ 30″	2′ 50″	3′ 7″

killed by the action of the serum, the serum introduced into the heart may or may not be inactivated.

Exp. 27. The heart of a non-pregnant female cat was isolated from the general circulation by ligatures. 1 cc. of fresh serum was injected into each ventricle. Twelve minutes later a few drops of blood were withdrawn from each ventricle and added to oxalated plasma. The blood from each ventricle rapidly clotted the oxalated plasma, coagulation commencing in less than 2′ 30″. Fifteen minutes later the animal was killed and *post mortem* examination showed the absence of clots from the ventricles.

Exp. 28. The experiment described above was repeated with a male cat and gave similar results.

Exp. 29. In another male cat 1 cc. of fresh serum was injected into each ventricle of the isolated heart. The heart stopped beating. Blood withdrawn from the right ventricle did not clot oxalated plasma but that withdrawn from the left ventricle produced a filamentous clot in oxalated plasma. There was slight intracardiac clotting in the right ventricle but the blood in the left ventricle was completely fluid.

A marked difference in the resistance of intracardiac blood to coagulation *in vivo* is apparent when fresh blood-serum is injected into the isolated hearts of pregnant cats, even when relatively small amounts of serum are used. Massive clotting may be produced in one ventricle whilst the blood remains fluid in the other ventricle. This may be partly due to changes in the equilibrium of the plasma-colloids since the content of fibrinogen and of lipoids as well as the suspension stability of the blood is altered in pregnancy, and one of us (J. W. P.) has frequently observed hypercoagulability of the blood of women in an advanced stage of pregnancy.

Exp. 30. Blood was obtained from the carotid artery of a cat in an advanced stage of pregnancy. Clotting times on glass at 19° were: commencement of coagulation 2′ 15″, completion of coagulation 6′ 40″. Hypercoagulability was thus manifested by the rapidity of the inception of clotting. The heart was isolated from the general circulation and 0·5 cc. of fresh serum was rapidly injected into each ventricle. Eight minutes later blood was withdrawn from each ventricle. The blood from the left ventricle completely clotted oxalated plasma in 6′ 30″, that from the right ventricle in 8′ 30″. *Post mortem* examination showed a massive clot and some fluid blood in the right ventricle and no clots in the left ventricle. The fluid blood from both ventricles clotted when shed.

VI. *The behaviour of fresh tissue juices in relation to extravascular and intravascular clotting.*

The experiments numbered 31–36 show that freshly prepared tissue juices obtained by crushing the lining of the aorta, testes, muscles or kidneys, rapidly induce coagulation in pure blood shed into paraffined vessels. Exps. Nos. 37–40 illustrate that relatively large amounts of the fresh juices of testes, muscles or kidney may be injected into either the general circulation or into the heart isolated from the circulation of non-pregnant cats without producing intravascular clotting. Exp. No. 41 demonstrates that the fresh juice of the kidney may induce localised intravascular coagulation in the pregnant cat. A fundamental difference is thus apparent in the resistance of unshed and shed blood to coagulation by tissue juices, which cannot be ascribed to neutralisation of

the coagulant material by an anticoagulant either secreted by the liver or liberated from the cells of the intima of the aorta, since these influences are excluded in Exp. No. 38, and Exp. No. 31 shows that the fresh juices of the aortic lining hasten the coagulation of the blood. It is suggested that the absence of coagulation *in vivo* from the non-pregnant animal is due to the specific resistance of the plasma complexes to dissociation by disturbing material and that this resistance is lowered in pregnant animals (*cf.* Exp. No. 30).

Table VII. *Illustrating the rapid coagulation of pure blood shed into paraffined vessels after the addition of fresh tissue juices.*

Temperature 19°.

No. of exp. ...	31	32	33
	5 cc. of blood of non-pregnant female + 0·05 cc. of juice from aortic intima	2·5 cc. of blood from a male + 0·05 cc. of testis juice	5 cc. of blood from a male + 0·05 cc. of testis juice
Commencement of clotting	4′ 0″	—	1′ 50″
Completion of clotting	5′ 15″	0′ 5″	2′ 40″

No. of exp. ...	34	35	36
	2·5 cc. of blood of non-pregnant female + 0·05 cc. of muscle juice	2·5 cc. of blood of non-pregnant female + 0·05 cc. of kidney juice	5 cc. of blood of pregnant female + 0·1 cc. of kidney juice
Commencement of clotting	2′ 30″	2′ 40″	—
Completion of clotting	5′ 45″	3′ 0″	1′ 10″

These observations are consistent with those of Morse [1919], who obtained material which hastened blood clotting from scrapings of the aorta, and with those of Mason [1924] who found that fresh extracts of the aortic intima as well as those of brain, kidney, lung, liver, testes and spleen accelerated the coagulation of oxalated plasma after "recalcification." They are also consistent with the finding of Pickering and Hemingway [1926] that extracts obtained from the tissues of the brain and lungs accelerate the clotting of blood shed into paraffined vessels.

Table VIII. *Illustrating the results obtained by the rapid intravascular injection of the fresh juices of testis, muscle and kidney.*

(10 cc. of tissue juice were injected into the jugular vein in each experiment except in No. 38, in which 0·5 cc. of testis juice was injected into each ventricle of the heart after isolating the heart from the general circulation by ligatures.)

Exp. 37	Exp. 38	Exp. 39	Exp. 40	Exp. 41
♂ (4½ kg.) testis juice injected. No intravascular clotting	♂ Testis juice injected. No clotting in the ventricles of the heart	♀ (2½ kg., non-pregnant). Muscle juice injected. No intravascular clotting	♀ (4 kg., non-pregnant). Kidney juice injected. No intravascular clotting	♀ (4 kg., in advanced stage of pregnancy). Kidney juice injected. Massive clots in portal vein

The normal blood volume of the cat has not yet been accurately estimated but may be assumed to amount approximately to 50 cc. of blood per kg. of body weight. A comparison of Tables VII and VIII shows that concentration of tissue juices employed *in vivo* were much greater than those that sufficed for the rapid and complete coagulation of blood shed into paraffined vessels. Larger doses could not be employed *in vivo* as respiration failed and the heart ceased beating. It may be added that an increase of the amounts of tissue juice added to shed blood, beyond those noted in Table VII, accelerates extravascular clotting on a paraffined surface.

There was no indication of a negative phase of coagulation in any experiments either *in vivo* or on shed blood. In Exp. No. 41 the blood remained fluid in all the larger vessels except in the portal vein, and was also fluid in the right ventricle of the heart. The left ventricle, the auricles and aorta were empty. Other pregnant animals showed intravascular clotting limited to the veins in the abdomen or to the left ventricle of the heart and like results were obtained with muscle juice.

A sharp contrast is apparent between the maintenance of the fluidity of the blood in non-pregnant cats after the rapid intravascular injection of massive amounts of the fresh juices of kidney and testes and the extensive intravascular clotting which follows the intravascular injection of extracts of these organs, which have been prepared by the methods of Wooldridge [1886, 1888, 1893] or of Halliburton [1891]. In the former technique strong acidification is employed, in the latter strong solutions of common salt are used. Both these procedures may involve either the dissociation of the colloidal complexes of tissue material or the denaturation of proteins, and in this connection the suggestion of Mason [1924] should be noted that the coagulants of tissue are normally united with protective colloid which is dissociated from the coagulant fraction during blood clotting. The repetition of precipitation and re-solution used in the "purification" of the extracts might remove a protective colloid, and so enhance the activity of the disturbing material.

VII. *The mode of action of thrombokinase and of thrombin in blood clotting.*

More than two hundred experiments were performed in an attempt to obtain sufficient information to decide whether the material liberated by the disintegration of blood platelets or from crushed tissues, known as thrombokinase in this country, and the participant in blood clotting called thrombin (the thrombase of Anwyl-Davies and Mellanby [1923]) react as enzymes or quantitatively in blood clotting. A conclusive answer to these questions was not found, but some light is thrown on them. There was no indication that the material released by the complete lysis of all the platelets in the blood hastens blood coagulation either *in vivo* or in blood shed into paraffined vessels (*vide* Exps. 10–15). It thus appears that the débris of blood platelets, when in a fresh condition, does not behave as an enzyme in blood which has not been disturbed by contact with a surface that it wets, and this conclusion is consistent with the observations of Fonio [1913] that massive doses of disintegrated blood platelets can be injected intravenously without producing intravascular clotting and with the medicinal use of this procedure without the occurrence of thrombosis.

It has already been noted, in Table IV, that blood in which the platelets have been disintegrated before shedding on glass clots more rapidly on glass than normal blood. But the decrease in the time required for the inception of clotting (2 mins.) is not greater than may be attributed to the almost instantaneous nature of the breakdown of the platelets. Our observations thus appear to be consistent with the conclusions of Bordet and Delange [1913] and of Bordet [1919, 1920] that quantitative reactions occur in the clotting of blood by the action of the débris of blood platelets.

The most cogent evidence that thrombokinase is an enzyme is provided by the observations of Mellanby [1917] on the coagulation of blood *in vitro* after the addition of testis extract; but no evidence is offered of identity in the behaviour of this material with that of the débris of blood platelets. It appears that conclusions based on these observations are valid only in the problem of the coagulation of shed blood after admixture with tissue juices and the experiments already illustrated in this paper suggest that a much wider range of experimentation is necessary before deciding whether the inception of intravascular and extravascular clotting can be ascribed to the same cause.

There was no indication in any of our experiments that fresh tissue juices inaugurate intravascular clotting by enzyme action. Much larger amounts of the fresh juices of testis, kidney and muscle than those required to produce rapid and complete clotting in shed blood were introduced into the blood-stream or into intracardiac blood without producing clotting in non-pregnant cats and the limitation of intravascular clotting to the blood of the abdominal veins or of the cardiac ventricles of the pregnant animals suggests local variations in the condition of the blood, rather than the occurrence of enzyme action (examples of the experiments are given in Table VIII).

The very rapid coagulation of blood shed into paraffined vessels after the addition of very small amounts of fresh tissue juices, illustrated in Table VII, suggests, however, catalytic action and these observations are consistent with the conclusions of Mellanby in so far as they apply to the inception of coagulation in shed blood by the action of tissue juices. But these experiments do not discredit the alternative explanation offered by Mills [1928] that enzyme action is simulated in blood clotting by a progressive release of the participants in clotting by the coagulation of the fibrinogen of the fibrinogen-prothrombin complex of the plasma.

Many attempts were made to confirm the statement of Rettger [1909] that quantitative reactions occur in the coagulation of the blood by the addition of fresh serum, but without any success. The experiments illustrated in Table VI show that very rapid and complete clotting follows the addition of minute amounts of fresh serum to blood shed into paraffined vessels. In only one experiment was there any indication which suggested a quantitative reaction.

Exp. 42. A medium sized blood clot was dropped into 2·5 cc. of blood shed into a paraffined vessel. Two minutes later the clot had doubled in size but the rest of the blood remained

completely fluid for nearly an hour. Coagulation commenced on the surface of the blood exposed to the air in 54 mins. and was complete in 65 mins. The control blood commenced to clot in 55 mins. and its clotting was complete in 65 mins.

It might be assumed that the thrombin of the serum in the blood clot was used chemically in the production of the increase in size of the clot but it appears equally probable that the thrombin was inactivated by adsorption on the surface of the clot. A study of the experiments of Rettger suggests that weak sera were used in his experiments and it appears probable that the explanation just offered accounts for the results he obtained. A large number of attempts were also made to obtain evidence of the alleged quantitative reaction in the coagulation of oxalated and citrated plasma by fresh serum. Reactions which simulated combination of the serum and the plasma were obtained but it was also apparent that fresh serum is inactivated by exposure, for 2–5 minutes, to 0·1 % potassium oxalate and a like reaction occurs after exposure to citrate. Inactivation of the serum thus accounts for the incompleteness of the coagulation when small amounts of serum are used. The experiments we have performed are thus consistent with the conclusion that fresh serum reacts as a catalyst in the clotting of shed blood, but they do not discredit the alternative explanation offered by Mills and mentioned above.

VIII. *The significance of the experiments in the problem of the maintenance of the fluidity of the blood.*

The experiments already described show that much larger amounts of fresh serum than are required to produce extravascular blood clotting can be injected into the hearts of non-pregnant cats, after the isolation of the heart from the general circulation, without producing intracardiac clotting (*vide* Exps. Nos. 27, 28), and a like resistance *in vivo* is displayed to the inception of intracardiac clotting by the action of fresh tissue juices (Exp. 38). The isolation of the heart from the general circulation precludes the inactivation of either the thrombin of the serum or of the thrombokinase of the tissue juices by the hepatic secretion of an excess of anticoagulant, and the confirmation of the conclusions of Morse [1919] and of Mason [1924] that the lining of the aorta contains a very active coagulant discredits the suggestion of Popielski [1913] that the fluidity of circulating blood after the intravascular injection of a coagulant is maintained by the release of an anticoagulant from the vascular endothelium. Moreover, the demonstration of the presence of active thrombin in the blood aspirated from the heart after the intracardiac injection of fresh serum (*vide* Exp. 27) indicates that the fluidity of the blood can be completely maintained *in vivo* without the inactivation of thrombin.

A specific resistance of plasma-colloids to dissociation and coagulation *in vivo* is indicated rather than the maintenance of the fluidity of the blood by the ingress of anticoagulants into the blood-stream. It has been pointed out by Pickering [1928] that blood-plasma possesses only a limited capacity of resisting disturbance and he suggests that changes in the surface condition

of shed blood lead to the dissociation of plasma-colloids whereby material in equilibrium is replaced by bodies which are susceptible to rapid change. On this assumption, the lack of resistance of shed blood to coagulation by fresh serum, by the material liberated by the lysis of platelets and by that present in fresh tissue juices becomes intelligible.

IX. *The significance of the experiments in the problem of natural thrombosis.*

Thrombosis has been defined as intravascular clotting arising during life-time. This definition is, however, inadequate, since the most simple forms of thrombi do not contain blood clots. But both red and mottled thrombi are largely composed of clotted blood, and red thrombi display a similar histological structure to that of the intravascular clots produced by the intravenous injection of tissue extracts [Mason, 1924]. An intimate relationship between natural thrombosis and experimental intravascular clotting thus seems apparent. The great resistance displayed by both male and non-pregnant female cats to intravascular clotting by fresh tissue juices suggests that ingress of such bodies into the blood-stream after trauma does not explain post-operative thrombosis, whilst the production of intravascular clotting in pregnant cats, limited to veins in the abdomen or to the left ventricle of the heart, by the intravenous injection of fresh tissue juices, suggests that localised variations in the condition of the venous blood occur during pregnancy, which conduce to thrombosis.

SUMMARY.

1. The disintegration of leucocytes is not an essential occurrence in the inception of blood clotting.

2. The complete disintegration of blood platelets neither produces intravascular clotting nor hastens the coagulation of normal blood which has been shed into paraffined vessels.

3. The lysis of blood platelets hastens the clotting of blood shed on to glass and also hastens the coagulation of blood shed into paraffined vessels when the blood is contaminated with tissue juices.

4. Fresh blood-serum promptly clots both pure blood and pure blood-plasma which have been shed into paraffined vessels, but the rapid intravascular injection of amounts of fresh sera sufficient to produce extra-vascular clotting does not produce intravascular coagulation in non-pregnant cats, if the amount administered is not lethal.

5. The rapid injection of fresh blood-serum into the ventricles of the hearts of pregnant cats, after the isolation of the heart from the general circulation, may or may not produce intracardiac coagulation of the blood.

6. The addition of very small amounts of the fresh juices of testis, muscle and kidney to blood shed into paraffined vessels rapidly produces complete coagulation, but the rapid intravascular or the rapid intracardiac injection (after the isolation of the heart from the general circulation) of these juices does not produce clotting *in vivo* in non-pregnant cats.

7. The production of intravascular clotting by the intravenous injection of the fresh juices of kidney and muscle into pregnant cats is described.

8. The significance of the data recorded in the explanations of the inception of the coagulation of the blood, of the maintenance of the fluidity of the blood *in vivo* and of the genesis of natural thrombosis are discussed.

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CV. THE CHEMICAL NATURE OF VITAMIN C.

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(*Received April 25th, 1932.*)

Hexuronic acid was discovered [1927] and isolated from plants and the adrenal cortex [1928] by Szent-Györgyi at the Biochemical Laboratory, Cambridge, and was investigated in regard to its function and chemistry [1928, 1930, 1931]. Its chemical configuration has been studied by Haworth, Hirst and Reynolds [1932]. The striking similarity of this substance with vitamin C was evident from the beginning. As far as it is known, its distribution in plants closely follows the distribution of the vitamin. The chemical properties of both substances agree and both are biologically active in minute quantities.

As is known, hexuronic acid has strongly reducing properties and the reducing power of many plant extracts is mainly due to this substance. The close relation between vitamin content and reducing power has been emphasised by Tillmans *et al.* [1932], whose careful work strongly suggested that the reducing power of plant extracts was in part due to the vitamin itself, which was identical with hexuronic acid.

Tillmans and his co-workers claim to have shown that the vitamin content and the reducing capacity run on strictly parallel lines under many conditions. Furthermore they have shown that the reducing factor can be oxidised in two different ways, reversibly and irreversibly. They have succeeded in reversing the oxidation of the reversible type by the application of reducing agents such as H_2S. They observed that by the reversible oxidation the reducing factor becomes very labile and prone to undergo irreversible changes. The vitamin showed a closely analogous behaviour. On treatment with oxidising agents, which produce a reversible oxidation of the reducing factor, the vitamin did not lose its activity, but became very labile. Tillmans and his collaborators were led to the conclusion that the reducing factor and the vitamin were in fact identical.

As is known, hexuronic acid can be oxidised reversibly and irreversibly, the reversible form being readily again reduced by H_2S. The agents reported by Tillmans to produce a reversible change in reducing factor also oxidise hexuronic acid reversibly. Agents, such as molecular oxygen, which produce an irreversible change in the reducing factor, also destroy hexuronic acid.

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The strongly reducing properties of active fractions of lemon juice were also emphasised by King and his co-workers [King and Waugh, 1932; Smith and King, 1931; Svirbely and King, 1931].

In a series of his well-known publications also, Zilva [1927, 1928, 1930] has found many parallels in the distribution of vitamin and reducing factor. Zilva, however, partly in collaboration with Connell [1924], was able to show that reducing capacity and antiscorbutic activity can vary independently during the course of fractionation. He succeeded in preparing fractions with a high antiscorbutic activity and no reducing power as well as fractions with a marked reducing capacity and no antiscorbutic activity. From this he justly concluded that vitamin C and the reducing factor could not be identical. Zilva also observed that on oxidising the reducing factor the antiscorbutic activity did not disappear, but the antiscorbutic principle became very labile, in which observation he was corroborated later by Tillmans. Zilva explained this change in stability by a protecting action of the reducing factor.

The reversible oxidation of hexuronic acid admits the possibility that in Zilva's extracts, which were antiscorbutically active but showed no reducing power, the hexuronic acid was present in a reversibly oxidised state. On the other hand it is known that the reducing factor is of a complex nature, as was also lately brought out by Guthrie and Wilcoxon [1932]. One of us (A. Sz.) has spent much time in studying a phenolic substance, found in relatively great quantities in orange juice, which shows a marked reducing power. It shows similarities to the flavonols but also gives reactions different from this group of substances. This substance is also readily carried down by lead acetate. Some years ago it was kindly tested by Zilva for antiscorbutic activity with negative results. The presence of such a reducing substance, not identical with hexuronic acid, might explain the reducing power of fractions which show no antiscorbutic activity. Taking all this into account, the authors think that Zilva's evidence against the identity of hexuronic acid and vitamin C cannot be regarded as conclusive.

Another objection raised recently by Zilva [1932] against the identity of vitamin C and hexuronic acid is based on the magnitude of the protective dose of hexuronic acid. The protective daily dose of lemon juice is 1·5 cc., which would keep growing guinea-pigs entirely free from scurvy. This quantity of lemon juice contains approximately 0·5 mg. of hexuronic acid. The protective dose of this substance would be thus of this magnitude. Zilva points out that with 0·5 mg. of fractions of lemon juice, which were still grossly contaminated, animals were protected against scurvy by himself and other authors. Thus the vitamin must have a higher potency.

The authors are of opinion that this evidence is not conclusive. It is difficult to judge to what extent these fractions were contaminated, and how much hexuronic acid they contained. If, however, 0·5 mg. of hexuronic acid gives full protection against scurvy, it is natural that somewhat smaller doses should have some activity. On the other hand 0·5 mg. of hexuronic acid for

1·5 cc. lemon juice gives only an approximate value, and it is possible that the hexuronic acid content of lemon juice is somewhat lower. Owing to the lack of a specific reaction it is impossible to state at present to what extent the reducing activity of a plant extract is entirely due to hexuronic acid or other substances and only rough estimates can be given.

We think that no circumstantial evidence can have much value either way in a question which can be subject to direct experiment. If it can be shown that the hexuronic acid present in the lemon juice has the same activity as the lemon juice itself, there can be little doubt about the identity of vitamin and hexuronic acid.

The striking similarity of vitamin C and hexuronic acid has been evident ever since the discovery of the acid. The possible identity of both substances has been expressed repeatedly by one of us. The collaboration of the present authors at the Institute of Medical Chemistry of the University of Szeged offered the necessary facilities for the testing of the antiscorbutic activity of hexuronic acid. The experiment was undertaken last autumn with a positive result. The negative controls died with symptoms of severe scurvy, while the animals receiving hexuronic acid were kept alive and free from scurvy in a 56 days' test. Owing however to the small number of animals used and certain deficiencies in the diet, which prevented normal growth, the results of the test were not published. A short account of this test was given in our first preliminary note [1932].

In the test to be reported[1], all shortcomings of the first experiment were remedied. The authors think that the experiment shows clearly that the hexuronic acid present in the lemon juice has the same activity as lemon juice itself, and thus clearly brings out the identity of the two substances[2].

Experimental.

The general procedure used in testing the antiscorbutic activity of hexuronic acid was that recommended by Sherman *et al.* [1922]. In addition to the basal diet, 0·5 cc. of cod-liver oil was given to each test animal every week.

The hexuronic acid used in the test was prepared in crystalline form from ox suprarenal glands 2 years ago at the chemical department of the Mayo Foundation at Rochester. We wish to emphasise the fact that our preparation was derived from animal tissues. If our hexuronic acid had been prepared from lemon juice or other plant sources the objection might arise that the activity was not due to the acid itself but to a contamination by a more potent antiscorbutic substance.

[1] A brief account of this experiment was given in our two preliminary notes in *Nature*.

[2] Simultaneously with our preliminary note King and Waugh [1932] reported that they had obtained crystals from lemon juice, which showed antiscorbutic activity and were apparently similar to a hexuronic acid. Since no chemical analysis was reported the chemical nature of their product is left in doubt. Since no mention was made of weight curves, number of animals, duration of the experiment and *post mortem* examinations, their claim of an antiscorbutic activity, based on "vitality," cannot be judged.

A sufficient amount of the acid was weighed out every 8 days and dissolved in water. Aliquot portions of the solution, adequate for daily feeding, were put in small phials and kept at $-18°$ in an atmosphere of CO_2. The solution was administered to the animals by a pipette. The minimum protective dose of lemon juice is generally regarded as 1·5 cc., and this quantity contains approximately 0·5 mg. of hexuronic acid. In our test, however, 1 mg. of hexuronic acid has been given daily to the animals since, owing to the age of the preparation and the unavoidable exposure to air, some of the acid may have become decomposed.

There was only one disturbing factor in our experiment, which however did not essentially influence the result. Owing to the general economic conditions we were unable to obtain milk powder until the 19th day of the experiment, at which time it was introduced into the diet. It was due to this fact that until that day the animals did not show normal growth, which however was promptly attained as soon as the milk powder was given. This growth was not due to traces of vitamin present, since the skimmed milk powder was heated in a tray in the drying-oven at 110° for 4 hours. It was specially tested in two animals on the 43rd day of the main test (see Table I and Fig. 1).

Table I.

Fed daily	No. of animals	Average survival (90 days' test) days	Average scurvy score*	Average gain in weight g.
1 mg. of hexuronic acid	3	55†	0	41
1 cc. of lemon juice	2	55†	2	81
Basal diet only	9	26	15	−103
1 mg. of hexuronic acid	7	90	0	281
1 cc. of lemon juice	6	90	5	147
Basal diet only	2	30	19	−114

* Highest possible score is 24. † Chloroformed.

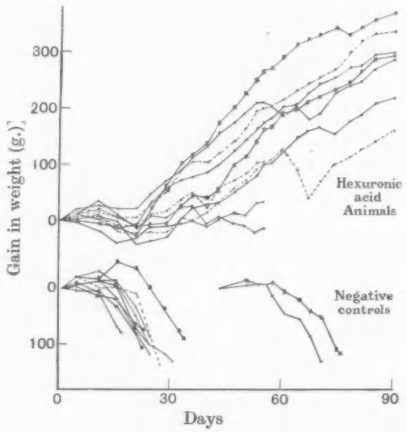

Fig. 1.

The following groups of test animals were used: (1) those receiving 1 mg. of hexuronic acid daily; (2) the positive controls, which received 1 cc. of lemon juice daily; (3) the negative controls, which received the basal diet only.

The results of the test are given in Table I. In Fig. 1 the weight curves of the animals receiving hexuronic acid are contrasted with those of the negative controls; Fig. 2 gives the average weight curves of the three groups of animals used in the test.

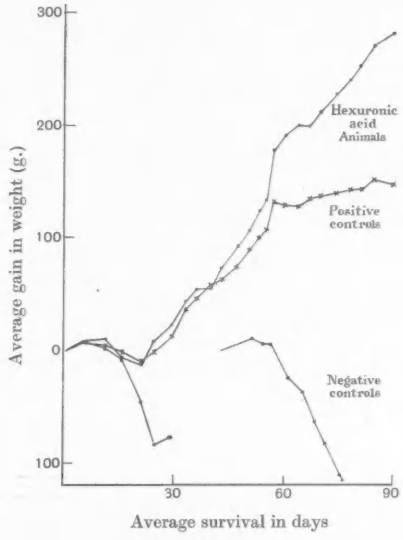

Fig. 2.

As seen from the Table and Figs. all the negative controls died within the period of 20–34 days with the usual loss in weight. At autopsy they all showed symptoms of severe scurvy. The animals receiving hexuronic acid showed normal growth. Three of these animals were killed at the end of 55 days and the rest at the end of 90 days' test period. At autopsy they were found entirely free from symptoms of scurvy. The positive controls receiving 1 cc. lemon juice showed mild scurvy at autopsy.

Summary.

Details of an experiment are described in which hexuronic acid was tested for antiscorbutic activity. The acid was administered in doses comparable with the hexuronic acid content of the protective dose of lemon juice. Given in this quantity (1 mg. daily), hexuronic acid affords complete protection against scurvy in a 90 days' test. Since it is generally assumed that animals kept free

from scurvy for 90 days would remain so indefinitely under identical conditions, it is concluded that vitamin C is a single substance and identical with hexuronic acid.

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CVI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. X.

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Owing to the difficulty of isolating vitamin C attempts were made in the present series of investigations to obtain some evidence of the chemical nature of this vitamin indirectly by studying the mode of its inactivation under various conditions. A fact observed some time ago in this connection was that when decitrated lemon juice was autoclaved for 1 hour under anaerobic conditions [Zilva, 1927] the reducing principle and vitamin C in the juice disappeared more rapidly than they did in un-autoclaved juices. Further experiments [Zilva, 1929] produced suggestive evidence that a substance, possibly of a phenolic character, was formed in the process of autoclaving which conduced to the increased rate of destruction of the reducing principle and the antiscorbutic factor.

In the experiments to be described we made an endeavour to throw further light upon this phenomenon.

We have now been able to show that when the reducing sugars (invert sugar [Zilva, 1924]) were removed from the juice by anaerobic fermentation, the spontaneous inactivation of the reducing principle or of vitamin C was no longer accelerated by the process of autoclaving. Further, when solutions of glucose, fructose or invert sugar containing calcium citrate, Rochelle salt, sodium carbonate or dipotassium hydrogen phosphate were autoclaved under the same conditions as the decitrated lemon juice they acquired the property of accelerating the destruction of the reducing principle and vitamin C. This to our mind proves conclusively that the factor formed in the process of autoclaving which increases the rate of inactivation of the vitamin complex is formed from the sugar present in the lemon juice.

Attempts to isolate and identify this substance did not meet with success owing to the very small quantities formed. Nevertheless from a comparison of the chemical properties of the ethereal extracts of autoclaved sugar solutions and autoclaved decitrated lemon juice with those of the di- and trihydric phenols, satisfactory evidence was obtained that a substance or

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substances resembling catechol or some of its derivatives was formed as the result of autoclaving.

In the course of this work it was also observed that dihydric phenols were unable to accelerate the destruction of the reducing principle in the absence of an enzymic factor though their oxidation products such as quinone *etc.* could do so unaided. The "thermostable peroxidase" [Zilva, 1929] does not seem to take any part in this mechanism of inactivation.

The preparation and properties of the ethereal extract of autoclaved decitrated lemon juice.

Method of extraction. Decitrated lemon juice adjusted to p_H 7 was autoclaved under anaerobic conditions for 1 hour at 40 lbs. pressure as described in a previous communication. After cooling and filtering, the reaction was again adjusted to p_H 7 and the juice shaken for 30 mins. in a mechanical shaker with $1\frac{1}{4}$–$1\frac{1}{2}$ volumes of peroxide-free ether. Batches of 200 cc. of juice were extracted in stoppered bottles of 600 cc. capacity, the air being displaced by CO_2 prior to shaking. The ethereal layer after separation was first concentrated at 35° under reduced pressure to a small volume and was finally dried in an evacuated desiccator over sulphuric acid. A yield of about 0·2 g. of a black-brown, tarry residue was thus obtained per litre of juice. A second extraction of the juice did not remove any appreciable further quantity nor was there any improvement in the yield when the juice was extracted by a continuous method for periods ranging from 3 to 5 hours.

Solubility. The residue, after being dried, redissolved in water or ether only with difficulty, the aqueous solution being neutral to litmus. It was soluble in ethyl or methyl alcohol, acetone and glacial acetic acid. It dissolved in dilute alkali carbonate and alkalis giving a brown solution and in sulphuric acid with a red colour. It was not reprecipitated from alcoholic solution by ether but the addition of water caused a turbidity.

Chemical properties. It contained only traces of nitrogen, but no phosphorus, sulphur or halogens. It decolorised both acid and alkaline $KMnO_4$ immediately in the cold and absorbed bromine and iodine from aqueous solutions. It reduced ammoniacal $AgNO_3$ instantly, but Fehling's solution only slowly in the cold and gave a Prussian blue precipitate with a mixture of $FeCl_3$ and K_3FeCy_6. A precipitate was formed in the aqueous solution on the addition of bromine. With $FeCl_3$ it gave a brown coloration which turned red upon the addition of a weak alkali, a reaction resembling that of catechol compounds. Tests for peroxide, with starch and KI, $K_2Cr_2O_7$ and titanium sulphate, were negative even after solutions of the residue had been kept in air for several days.

The properties of autoclaved sugar solutions and the preparation and properties of their ethereal extracts.

In attempting to trace the constituent of decitrated lemon juice from which this ether-soluble substance was formed, attention was focused upon the sugar content of the juice, because of the work of Hoppe-Seyler [1870], Bernhauer and Nepp [1931] and others who have shown that phenolic substances are formed on heating sugars and carbohydrates at high temperatures in the presence of alkalis and also because of the following observations made by ourselves. We found that when the reducing sugar in decitrated lemon juice was fermented the iodine-absorbing capacity of the juice did not increase on autoclaving and, further, the addition of such autoclaved juice to ordinary decitrated lemon juice did not accelerate the destruction of the reducing principle as does that of an autoclaved unfermented juice. This is shown by the following experiment. 200 cc. of decitrated lemon juice at p_H 7·0 together with 5 g. of calcium carbonate were placed in an ampoule which was then exhausted and kept at 38°–40° for 24 hours. After this time practically the whole of the invert sugar had been spontaneously fermented. The solution was then filtered and the filtrate autoclaved under strictly anaerobic conditions for 1 hour at 143° [see Zilva, 1928]. 100 cc. of the autoclaved solution after cooling and filtering were evaporated to dryness under reduced pressure at 40° and the residue dissolved in 100 cc. of untreated decitrated lemon juice. A batch of decitrated lemon juice which had not been previously fermented was treated similarly. These two solutions as well as a control batch of the same decitrated lemon juice were stored aerobically in a cold room and titrated periodically with 0·02 % phenolindophenol solution. As will be seen from Fig. 1, the destruction of the reducing principle in the control and in the solution containing the residue from the autoclaved, fermented, decitrated lemon juice proceeded at the same rate, whilst in the decitrated juice containing the residue from the autoclaved, unfermented, juice, the disappearance of the principle was greatly accelerated.

The effect of autoclaving sugar solutions was therefore next studied. It was found that autoclaving 1 % solutions of glucose, fructose or invert sugar, (decitrated lemon juice usually contains about 1·0 % of invert sugar), did not cause them to absorb iodine in neutral solution, nor did the addition of their residues (obtained by evaporation under reduced pressure at 40°) to neutral decitrated lemon juice accelerate the destruction of the reducing principle in the juice on storage in air. This will be seen from Fig. 2. On the other hand, when 1 % solutions of these sugars containing 0·25 % of sodium carbonate, dipotassium phosphate, Rochelle salt or sodium citrate were autoclaved anaerobically for 1 hour at 143° they exhibited the same property of accelerating the inactivation of the reducing principle in neutral solution as autoclaved decitrated lemon juice (Fig. 2). The same result was obtained with an autoclaved 1 % invert sugar solution containing 8 % citric acid (lemon juice usually

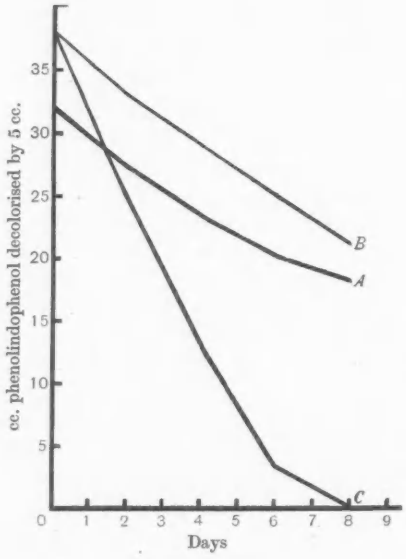

Fig. 1. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing residue from autoclaved fermented decitrated lemon juice; *C*, decitrated lemon juice containing residue from autoclaved decitrated lemon juice.

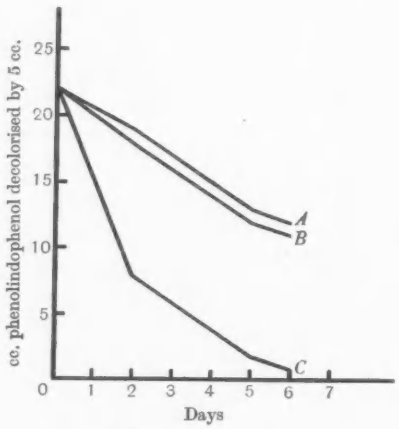

Fig. 2. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing residue from autoclaved 1 % invert sugar solution; *C*, decitrated lemon juice containing residue from an autoclaved solution of 1 % invert sugar–0·25 % sodium carbonate mixture.

contains about this amount of citric acid) which had been decitrated with calcium carbonate. An ethereal extract prepared from a 1·0 % invert sugar solution containing 0·25 % of sodium carbonate, which had been autoclaved, showed identical chemical properties with the ethereal extract obtained from autoclaved lemon juice described above. In this case the ferric chloride reaction for catechol compounds was unmistakable.

The addition of the residue from an autoclaved solution of a mixture of sugar and sodium carbonate to decitrated lemon juice also increased the rate of destruction of vitamin C. This is shown by the following biological experiment.

The residue from 100 cc. of an autoclaved solution of a mixture of 1 % invert sugar and 0·25 % of sodium carbonate, obtained by evaporation under reduced pressure at 40–45°, was dissolved in 100 cc. of decitrated lemon juice. This solution, as well as a control batch of the same decitrated lemon juice stored at neutral reaction for 5 days, was tested for its antiscorbutic potency. Four guinea-pigs on a daily dose of 3 cc. and 3 guinea-pigs on a daily dose of 5 cc. of the control juice showed no, or very mild, signs of scurvy at the autopsy when chloroformed after 60 days. In contrast to this 7 guinea-pigs receiving daily 3 cc. of the stored decitrated lemon juice containing the residue from an autoclaved solution of sugar-carbonate mixture and 5 guinea-pigs on a daily dose of 5 cc. of the same preparation died of the disease within 45–60 days. The addition of this residue to decitrated lemon juice is thus seen to produce the same effect upon the vitamin C of the juice as that produced by the addition of autoclaved decitrated lemon juice [Zilva, 1928, 1929].

A comparison of the chemical properties of the ethereal extracts with those of polyhydric phenols.

Various attempts have shown that the isolation from autoclaved decitrated lemon juice of the substance which accelerates the destruction of the reducing principle and of vitamin C is a task fraught with many difficulties. However, in view of the phenolic properties of the ethereal extracts of autoclaved decitrated lemon juice and sugar solutions, it was considered that a comparison of the chemical properties of these extracts with those of polyhydric phenols might yield some indirect information on the subject.

In addition to their ability to react with iodine in neutral solutions the ethereal extracts and the polyhydric phenols also react with iodine in alkaline solutions, iodoform separating during the reaction in each case. The quantitative nature of these reactions with iodine was therefore studied in detail.

The following phenols were investigated, catechol, guaiacol, homocatechol, protocatechuic acid, resorcinol, quinol, phloroglucinol, pyrogallol and hydroxyquinol.

The trihydric phenols and resorcinol and guaiacol were found as a result of preliminary experiments to be quite dissimilar in their chemical reactions from the ethereal extracts and were therefore not investigated further.

The reactions of the ethereal extracts with iodine. One litre of decitrated lemon juice at p_H 7·0 was autoclaved in the usual manner and after cooling and filtering was acidified with 7 cc. of 4*N* HCl. The juice was then extracted by shaking with 1200 cc. of B.P. ether for 30 min. as described earlier. The ethereal layer was separated, dried over $CaCl_2$, filtered and then evaporated to dryness under reduced pressure. The residue was dissolved as completely as possible with three lots of 7 cc. of methyl alcohol and finally made up at p_H 7·0 with water to 100 cc. An extract was prepared in the same manner from 1 litre of a solution containing 1 % invert sugar and 0·25 % Na_2CO_3 which had been autoclaved under the same conditions as the lemon juice. The solution of the extract from the autoclaved decitrated lemon juice was very turbid, whilst that from the autoclaved sugar solution was only slightly so. The material causing the turbidity could not be filtered off nor did it settle out as a sediment after standing several days.

Five cc. of each of these solutions were mixed with 10 cc. of *N*/100 I and, after standing 5 minutes, the unabsorbed excess was determined with *N*/100 sodium thiosulphate. The extract from the juice absorbed 3·6 cc. *N*/100 I and that from the autoclaved sugar solutions 3·8 cc. *N*/100 I. With these extracts a very rapid absorption of iodine occurred in 5 mins., further absorption took place very slowly, as will be seen from Table I. The reactions of iodine with the phenols, to be referred to later, were complete in 5 mins., when a suitable quantity of iodine was employed. We therefore assumed that the iodine absorption which took place during the first 5 mins. in the case of these extracts was comparable to that of the phenols.

Table I.

Solution of ethereal extract from	cc. of *N*/100 iodine used up by 5 cc. at neutral reaction in 5 min.	1 hr.	24 hrs.
Autoclaved decitrated lemon juice	3·6	4·4	6·2
Autoclaved solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture	3·8	4·7	7·6

Five cc. of each of these extracts were next mixed with 10 cc. *N*/10 I after which 1 cc. of *N* NaOH was added drop by drop with gentle shaking. The solutions after standing 10 min. were acidified with 1 cc. of 4*N* H_2SO_4 and the excess iodine determined with *N*/10 sodium thiosulphate. The extract from the autoclaved juice used up 2·8 cc. *N*/10 I whilst the other extract used 3·0 cc. *N*/10 I. Very small amounts of iodoform separated during both these reactions, which were found to be completed in the time employed.

The ratios between the amounts of iodine used up in the reactions in neutral solution and the amounts used up in the reactions in alkaline solution, *viz.* 1:7·8 in the case of the extract from the autoclaved juice and 1:7·9 in the case of the extract from the autoclaved sugar solution were found to be

fairly constant. This will be seen from Table II which gives data for extracts from six other batches of autoclaved lemon juice prepared in a similar manner and examined under the same conditions.

Table II.

Batch	Volume of autoclaved decitrated lemon juice from which extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 min. at neutral reaction	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 min. at alkaline reaction	Ratio A : B
1	250	100	1·0	7·6	1 : 7·6
2	500	100	2·1	16·7	1 : 8
3	500	100	5·1	40·0	1 : 7·8
4	150	100	1·1	8·0	1 : 7·7
5	150	100	0·5	4·4	1 : 8·8
6	150	100	0·9	7·7	1 : 8·5

It has been pointed out earlier that the material extracted by ether from autoclaved juices is not easily or completely redissolved in water but that it is readily soluble in alkalis or alkali carbonates. In cases where this latter fact was made use of in preparing aqueous solutions of the ethereal extracts instead of dissolving them in methyl alcohol or directly in water, the ratio between the amount of iodine absorbed in neutral solution in 5 mins. to that used up in alkaline solution tended to be constant also but at a lower value than in the former case. Solutions of five extracts were prepared by concentrating the ethereal extracts to small volume under reduced pressure, shaking the concentrated ethereal solution with 3 or 4 successive quantities of N Na_2CO_3 (5–7 cc.) neutralising the carbonate solution to p_H 7·0 with H_2SO_4 and removing CO_2 and ether from solution by exhaustion.

The results of the iodimetric examination of these solutions are recorded in Table III.

Table III.

Batch	Volume of autoclaved decitrated lemon juice from which extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 min. at neutral reaction	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 min. at alkaline reaction	Ratio A : B
1	100	100	0·9	4·5	1 : 5
2	100	100	1·7	7·3	1 : 4·3
3	300	50	4·8	24·0	1 : 5
4	300	50	2·1	17·7	1 : 8·4
5	1000	100	3·8	16·0	1 : 4·2

When small amounts of Na_2CO_3 or $NaHCO_3$ were added to the neutral reaction mixtures of iodine and those extracts in purely aqueous solutions, the

amounts of iodine absorbed in 5 min. also increased with the consequent lowering of the ratio to a value approaching that observed above (see Table IV).

Table IV.

Batch	Volume of autoclaved juice or solution from which ethereal extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A_1 cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 minutes at neutral reaction	A_2 cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 minutes in the presence of 0·5 cc. M $NaHCO_3$	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 minutes at alkaline reaction	Ratio A_1:B	Ratio A_2:B
Decitrated lemon juice	1000	100	3·6	7·7	28	1:7·8	1:3·6
Solution of 1·0% invert sugar and 0·25% sodium carbonate mixture	1000	100	3·8	8·1	30	1:7·9	1:3·7

If in the reactions in neutral solution between iodine and the extracts which had been dissolved in carbonate solution, mineral acid was added immediately after the end-point was reached in the back titration with sodium thiosulphate, iodine was instantly liberated.

The reactions of phenols with iodine. $M/100$, $M/200$, $M/400$ and $M/1000$ solutions of quinol, catechol, homocatechol and protocatechuic acid were employed in studying the reactions of these polyhydric phenols with iodine. In the experiments in neutral solution using 5 or 10 cc. of these solutions and $N/100$ I in excess, consistent results could only be obtained with quinol. In the case of the more dilute solutions it was found that, providing twice the theoretical amount of iodine was employed, the reaction was strictly quantitative, two atoms of iodine reacting with one molecule of quinol yielding benzoquinone according to the equation

$$C_6H_4(OH)_2 + I_2 \rightleftharpoons C_6H_4O_2 + 2HI.$$

After the end-point in the back titration had been reached iodine separated slowly from these solutions until a condition of equilibrium was attained. The results of these experiments are shown in Table V.

Table V.

Solution of quinol	cc. of $N/100$ iodine used up in 5 min. by 10 cc. of quinol solution at neutral reaction	Theoretical amount of iodine required for reaction involving 1 molecule of quinol and 2 atoms of iodine cc. $N/100$
$M/100$	19·2	20·0
$M/200$	9·6	10·0
$M/400$	5·0	5·0
$M/1000$	2·0	2·0

In the case of catechol, homocatechol and protocatechuic acid the amount of iodine used up was less than that required for the reaction analogous to that between iodine and quinol. When, however, either Na_2CO_3 or $NaHCO_3$ was added to the solutions of these phenols during the reactions, preferably

in amounts molecularly equivalent to the amounts of phenol used, the reactions proceeded quantitatively according to the general equation

$$X.C_6H_3(OH)_2 + I_2 \rightarrow X.C_6H_3O_2 + 2HI$$
$$(X = H,\ CO_2H \text{ or } CH_3).$$

On acidifying these solutions with mineral acid very soon after the end-point in the back titration, iodine was immediately liberated.

The reactions in alkaline solution were complete in 10 min. and consistent results could be obtained with each phenol. When suitable amounts of iodine were used the quantities of iodine used up were found to bear a quantitative relationship to the amount of phenol employed. The amount of iodoform which separated was very small except in the case of quinol. The results of these experiments are recorded in Tables VI and VII.

Table VI.

Solution of quinol	cc. *N*/100 iodine used up in 10 min. by 10 cc. of quinol solution at alkaline reaction	Theoretical amount of iodine required for reaction involving 1 molecule of quinol and 12 atoms of iodine cc. *N*/10
M/100	11·5	12·0
M/200	5·7	6·0
M/400	2·9	3·0
M/1000	1·2	1·2

Table VII.

Solution	Amount of phenol solution used cc.	cc. of *N*/10 iodine used up in 10 min. at alkaline reaction	No. of atoms of iodine per molecule of phenol involved in reaction
M/100 catechol	5	3·0	6·0
M/100 homocatechol	10	8·0	8·0
M/100 protocatechuic acid	10	6·4	6·4

The ratios of the amounts of iodine used up by these phenols in the reaction according to the above general equation to the amounts used up in the presence of caustic alkali are:

quinol	1:6
catechol	1:3
homocatechol	1:4
protocatechuic acid	1:3·2

when the former reaction is quantitative, as in the presence of sodium carbonate or bicarbonate. Mention was made earlier that, in the absence of these salts, this reaction does not proceed quantitatively, except in the case of quinol. It would therefore be expected that in the case of catechol and its derivatives the above minimum values of these ratios would under certain conditions reach a value of 1:8, similar to that obtained with the ethereal extracts from autoclaved decitrated lemon juice and autoclaved solutions of invert sugar and sodium carbonate mixture.

The above experiments show that the general behaviour of these phenols during these reactions is similar to that of solutions of the ethereal extract and that the behaviour of the ethereal extracts approximates to that of catechol and its derivatives rather than to that of quinol. This circumstance coupled with the observation that the ethereal extracts give a catechol colour reaction with ferric chloride solution, among other typical reactions for phenols, suggests that a compound resembling catechol or its derivatives is formed from the sugars in the process of autoclaving.

The influence of enzymes on the inactivation of the reducing principle.

It was observed in connection with the instability of the reducing principle and vitamin C of autoclaved decitrated lemon juice [Zilva, 1929] that the treated medium as well as the original juice was capable of giving a reaction with *p*-phenylenediamine and H_2O_2. In consequence of this observation the existence of a "thermostable peroxidase" which had some influence on the inactivation of the two principles was postulated. An endeavour was now made to co-ordinate this phenomenon with the recent development of the subject.

In the following experiments 5 cc. of the solution to be tested, 1 cc. of 1 % *p*-phenylenediamine, 1 cc. of 1 % benzidine or 1 cc. of 10 % tincture of guaiacum and 4 or 5 drops of 6 % H_2O_2 were used. We found this strength of H_2O_2 convenient since in most experiments the solution contained reducing substances which destroyed this reagent. Blanks were used in all the tests.

Decitrated lemon juice, autoclaved decitrated lemon juice and autoclaved 1 % solutions of sugars were examined and the following facts were observed.

(*a*) They all gave a colour reaction with *p*-phenylenediamine but not with either benzidine or guaiacum tincture.

(*b*) The autoclaved solutions of pure sugars gave a colour reaction with *p*-phenylenediamine immediately, while the remainder responded only after a period of lag which varied from 15 to 40 min. It has been mentioned earlier that the autoclaved solutions of pure sugars as distinct from those containing sodium carbonate *etc.* do not contain iodine-absorbing substances. That this lag period was caused by the inhibiting influence of iodine-absorbing substances was shown by the fact that the lag period was reduced when the iodine-absorbing substances were removed either by precipitation with normal lead acetate (3 cc. of a mixture of 1 volume of saturated normal lead acetate solution and 4 volumes of water per 100 cc. of autoclaved sugar solution) or by adsorption with norite charcoal. In the case of the sugar solutions the iodine-absorbing substances were removed almost completely by the charcoal and the lag period thus reduced to zero. Typical results are shown in Table VIII.

It was found that this lag in the *p*-phenylenediamine-H_2O_2 reaction could be simulated by the addition of catechol to autoclaved solutions of the pure sugars.

(*c*) The ethereal extracts from autoclaved decitrated lemon juice and from an autoclaved solution containing 1 % sugar and 0·25 % sodium carbonate also give the *p*-phenylenediamine-H_2O_2 reaction after a period of lag.

Table VIII.

Solutions examined	cc. of $N/100$ iodine used up in 5 min. at neutral reaction by 5 cc. of autoclaved solution	Time in min. for colour to develop in *p*-phenylene-diamine-hydrogen peroxide test
1. 1·0 % invert sugar solution autoclaved 1 hr. at 143°	0·1	Colour developed immediately
2. Solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture autoclaved 1 hr. at 143°	7·9	10
3. Solution 2 after treatment with normal lead acetate solution	4·2	5
4. Solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture autoclaved 1 hr. at 145°	6·9	20
5. Solution 4 after treatment with norite charcoal	0·3	Colour developed immediately
6. Decitrated lemon juice autoclaved 1 hr. at 143°	8·2	15
7. Solution 6 after treatment with norite charcoal	1·9	0·5

(*d*) The ethereal extract from autoclaved decitrated lemon juice, which, as pointed out above, shows "thermostable peroxidase" activity, when added to decitrated lemon juice from which enzymes have been removed by precipitation with alcohol, increases the rate of destruction of the reducing principle when the mixture is stored aerobically at neutral reaction. This is shown by the following experiment. Decitrated lemon juice, p_H 7·0, was concentrated under reduced pressure at 45° to one-fifth of its original volume and was then diluted by the slow addition of 5 times its volume of 96 % alcohol. The filtrate was evaporated to a thin syrup under reduced pressure at 45°. The residue was made up to a convenient volume at p_H 7·0. Such a preparation showed no "peroxidase" activity and usually contained 75 to 80 % of the reducing capacity of the original juice as measured by the reduction of phenolindophenol.

An ethereal extract obtained from 100 cc. of autoclaved decitrated lemon juice and an amount of the above preparation equivalent to 100 cc. of the original juice were mixed and finally made up to 100 cc. at p_H 7·0 and stored in the cold room, together with a control solution of the "peroxidase-free preparation" of the reducing principle. The solutions were titrated at intervals with 0·02 % phenolindophenol. The result of this experiment is shown in Fig. 3.

(*e*) The residue obtained by evaporating under reduced pressure an autoclaved solution of 1·0 % invert sugar and 0·25 % sodium carbonate, which, as has been pointed out previously, contains both iodine-absorbing substances and the "thermostable peroxidase," increases the rate of destruction of the reducing principle in the above "peroxidase-free preparation" on storage in air. Some of the iodine-absorbing substances, which can be obtained free

from the "thermostable peroxidase" by precipitation with normal lead acetate solution produce the same effect without the aid of the "thermostable peroxidase." These observations are based upon the experiments described below in which the following preparations were used.

Preparation A. A solution of 1·0 % invert sugar and 0·25 % sodium carbonate was autoclaved as already described.

Preparation B. 100 cc. of preparation A were boiled for 2 min. with 2 g. of norite charcoal and then filtered. The filtrate was then shaken with 1 g. of charcoal and again filtered. The latter process was repeated giving a final almost colourless solution.

Preparation C. 3 cc. of normal lead acetate solution (one volume of saturated normal lead acetate solution mixed with four volumes of water) were

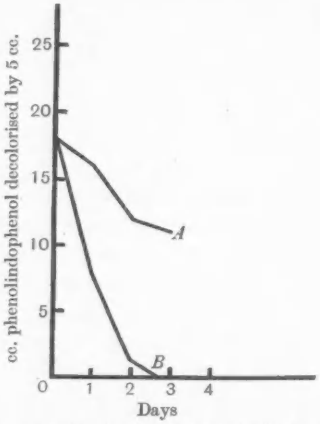

Fig. 3. *A*, "peroxidase-free preparation" of reducing principle; *B*, "peroxidase-free preparation" of reducing principle containing ethereal extract from autoclaved decitrated lemon juice.

added to 100 cc. of solution A and the precipitate was centrifuged off. This precipitate was suspended in 20 cc. of water and decomposed with 0·2 cc. of $4N$ H_2SO_4. The lead sulphate was removed on the centrifuge. The clear liquid was adjusted to p_H 7·0 and then diluted to 50 cc. with water.

Preparation D. The lead was removed as lead sulphate from the filtrate obtained in preparation C after precipitation with lead acetate. The solution was finally adjusted to p_H 7·0.

The preparations A, B, C and D were examined iodimetrically and for "thermostable peroxidase" with 1 % *p*-phenylenediamine solution and H_2O_2. The details are given in Table IX. (Solution C was diluted to half strength before samples were taken for test.)

Preparation E. A "peroxidase-free preparation" of the reducing principle was prepared as above from 400 cc. of decitrated lemon juice. The final solution measured 100 cc.

50 cc. of solutions A, B and D were concentrated under reduced pressure at 45°. Each was then mixed with 20 cc. of solution E, as was also 25 cc. of solution C and then each diluted to 50 cc. at neutral reaction. These solutions, together with 20 cc. of solution E diluted to 50 cc. with water as a control, were stored aerobically in the cold room and titrated periodically with 0·02 % phenolindophenol.

The results of this experiment are recorded in Fig. 4.

Table IX.

Preparation	cc. of *N*/100 iodine used up by 5 cc. of preparation in 5 min. at neutral reaction	Time in min. for colour to develop in *p*-phenylene-diamine-hydrogen peroxide test
A	4·9	10
B	0·3	Colour developed immediately
C	1·7	No development of colour occurred in 1 hour
D	2·2	3

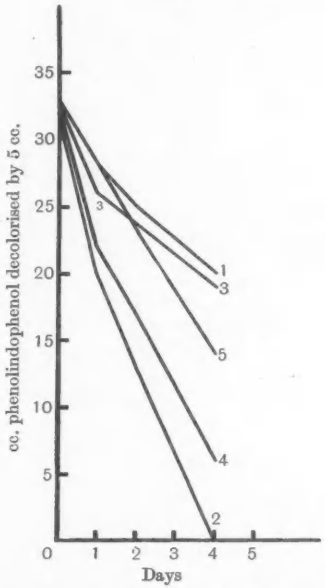

Fig. 4. 1, "peroxidase-free preparation" of reducing principle; 2, "peroxidase-free preparation" of reducing principle containing preparation A; 3, "peroxidase-free preparation" of reducing principle containing preparation B; 4, "peroxidase-free preparation" of reducing principle containing preparation C; 5, "peroxidase-free preparation" of reducing principle containing preparation D.

(*f*) Quinol, catechol, homocatechol and protocatechuic acid in 0·05 % concentration, whether alone or in the presence of the "thermostable peroxidase", do not increase the rate of destruction of the reducing principle in the above described "peroxidase-free preparation" of decitrated lemon juice

on storage in air whereas benzoquinone in this concentration destroys the reducing principle almost immediately under the same conditions. On the other hand these phenols accelerate the destruction of the reducing principle in this preparation in the presence of a true peroxidase, as for example that from turnip root, at about the same rate at which they increase the rate of its inactivation in decitrated lemon juice.

In these experiments 600 cc. of a "peroxidase-free preparation" of the reducing principle were prepared and one-half was used to dissolve the residue from 300 cc. of a 1 % solution of invert sugar which had been autoclaved as previously described. The residue, which contained the "thermostable peroxidase" but, as mentioned earlier, no iodine-absorbing substances, was obtained by evaporation under reduced pressure at 45°. 0·025 g. of quinol, catechol, homocatechol and protocatechuic acid were separately dissolved in 50 cc. of each of the above preparations. A "peroxidase-free preparation" of the reducing principle containing 0·05 % of benzoquinone was also included in this experiment. The solutions were then adjusted to p_H 7·0 and stored aerobically in the cold room. They were titrated at intervals with 0·02 % phenolindophenol. Table X records the results obtained.

Table X.

Solutions	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of test solution			
	1st day	3rd day	5th day	8th day
1. "Peroxidase-free" preparation of the reducing principle of decitrated lemon juice	22	19	17	14
2. Solution 1 containing 0·05 % of quinol	22	18	16	13
3. " " catechol	22	20	18	16
4. " " homocatechol	22	20	18	16
5. " " protocatechuic acid	22	22	17	15
		2nd day		
6. " " benzoquinone	30	0	—	—

Solutions	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of test solution			
	1st day	3rd day	5th day	8th day
1 A. "Peroxidase-free" preparation of the reducing principle of decitrated lemon juice containing "thermostable peroxidase"	22	18	17	14
2 A. Solution 1 A containing 0·05 % of quinol	22	16	14	12
3 A. " " catechol	22	17	16	15
4 A. " " homocatechol	22	16	14	14
5 A. " " protocatechuic acid	22	22	15	14

One-half of a "peroxidase-free preparation" of the reducing principle obtained from 400 cc. of decitrated lemon juice was mixed with 75 cc. of a solution of turnip root peroxidase and the mixture diluted to 150 cc. The preparation of the peroxidase solution is given below. The other half was diluted with water to 150 cc. In 50 cc. of each solution were dissolved 25 mg.

of quinol and 25 mg. of catechol respectively. The remainder served as controls. These solutions were then adjusted to neutral reaction and stored aerobically in the cold room. They were titrated periodically in the usual manner. Fig. 5 shows the results of these experiments. The peroxidase was still very active in all the solutions at the end of the experiments.

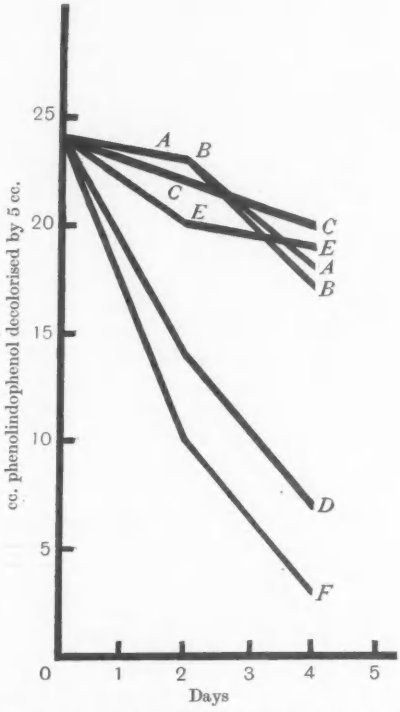

Fig. 5. *A*, "peroxidase-free preparation" of reducing principle; *B*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase; *C*, "peroxidase-free preparation" of reducing principle containing 0·05 % catechol; *D*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase and 0·05 % catechol; *E*, "peroxidase-free preparation" of reducing principle containing 0·05 % quinol; *F*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase and 0·05 % quinol.

The solution of the peroxidase was obtained from a frozen turnip root weighing 300 g. by removing the outer cortex while the root was still frozen and then slicing and mincing the remainder so that the pulp fell directly into an excess of alcohol in which it was allowed to thaw. The enzyme was then obtained by extracting the alcohol-free pressed tissue residue with water, the extracts being finally diluted to 200 cc. This enzyme preparation, which was neutral in reaction, gave no colour reaction with ferric chloride solution, did not reduce ammoniacal silver nitrate solution, Fehling's solution or phenol-

indophenol solution or absorb iodine from neutral solutions even after long standing. It was therefore considered to be free from phenolic substances. Four drops of this solution in 5 cc. of water gave very intense peroxidase reactions with 1 cc. of 1·0 % solutions of *p*-phenylenediamine, benzidine and 10 % tincture of guaiacum and H_2O_2.

(*g*) Although decitrated lemon juice does not contain a true peroxidase it seems to contain a thermolabile factor which catalyses the destruction of the reducing principle of the juice in the presence of polyhydric phenols. Thus when decitrated lemon juice at p_H 7·0 was steamed in ampoules under strictly anaerobic conditions for 1 hour the *p*-phenylene-diamine-H_2O_2 test was still given, as was to be expected, by this heated juice though the lag period had increased. 0·05 % of catechol or quinol was dissolved in such steamed juice and the solutions stored. Periodic titrations with phenolindophenol solution showed that the reducing principle was not destroyed at a greater rate in these heated juices containing the phenols, as it is in the case of unheated juice.

Steaming decitrated lemon juice under these conditions appears not to destroy the reducing principle to any appreciable extent nor is there any change in the iodine-absorbing capacity or in the condition of the reducing sugar.

The results of these experiments are shown in Fig. 6 and Table XI.

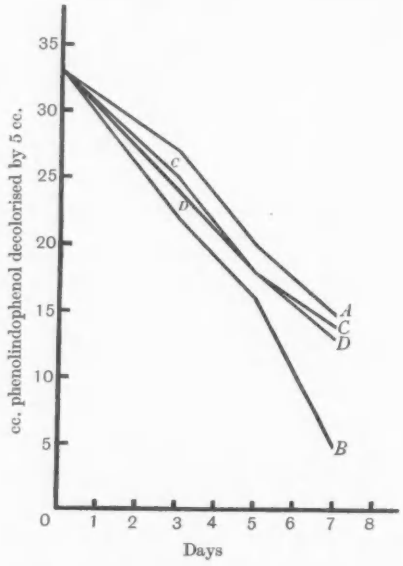

Fig. 6. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing 0·05 % quinol; *C*, decitrated lemon juice steamed 1 hour; *D*, decitrated lemon juice containing 0·05 % quinol steamed 1 hour.

Table XI.

Solution	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of juice	Amount of iodine expressed in cc. of N/100 sclution used up by 5 cc. of juice in 5 minutes at neutral reaction	Amount of reducing sugar expressed in mg. of glucose contained in 5 cc. of juice determined by Bertrand's method
Decitrated lemon juice	23	5·8	63
Decitrated lemon juice after heating in steamer for 1 hr.	21	5·7	63

Discussion.

The foregoing results prove clearly that the substance formed on autoclaving decitrated lemon juice which accelerates the inactivation of the reducing principle and of vitamin C on storage of the juice at neutral reaction originates from the sugar of the decitrated lemon juice. Further, the evidence obtained points rather convincingly to the fact that this substance is of the nature of a catechol compound. It is difficult to assert with precision from the available data the manner in which this substance actually inactivates the vitamin complex. We know however from observations described in earlier communications of this series that when quinol, catechol, resorcinol, benzoquinone or quinhydrone is added to ordinary unheated decitrated lemon juice, the same inactivating action can be obtained and it is therefore evident that this effect is produced by a great number of chemically related substances.

With regard to the "thermostable peroxidase," which was previously considered to be associated with the acceleration of the inactivation of the reducing principle and vitamin C [Zilva, 1929], the bulk of the evidence in this investigation indicates that this factor plays no part in the inactivating mechanism. Much more significant in this respect is the observation made that in decitrated lemon juice the reducing principle is destroyed on the addition of catechol and quinol whilst this destruction does not take place if the juice has been previously heated for 1 hour in a steamer. It is further of interest to note here that the dihydric phenols require the aid of some factor of an enzymic nature to effect this inactivation, whilst benzoquinone and the ethereal extracts of autoclaved decitrated lemon juice cause it unaided. The only reasonable assumption we may therefore make at this stage is that the phenolic hydroxy-compounds undergo intermediate oxidation and subsequently oxidise the vitamin complex.

Summary.

1. When the sugar of decitrated lemon juice is removed by anaerobic fermentation, the reducing principle and the antiscorbutic factor in the fermented juice are not inactivated at an increased rate after autoclaving and storing at neutral reaction, as they are in autoclaved unfermented decitrated lemon juice on storage.

2. Solutions of glucose, fructose or invert sugar containing certain salts, on being autoclaved under the same conditions as decitrated lemon juice, are

capable of increasing the rate of destruction of the reducing principle and vitamin C in decitrated lemon juice in neutral solution.

3. The chemical properties of the ethereal extracts of autoclaved decitrated lemon juice and of autoclaved solutions of sugar-salt mixtures have been studied. Both extracts are capable of accelerating the destruction of the reducing principle and vitamin C in decitrated lemon juice on storage.

4. It is concluded that the substance or substances which conduce to the acceleration of the inactivation of the above principle are produced from the sugar of the juice on autoclaving.

5. From a comparative study of the chemical properties of the ethereal extracts of autoclaved decitrated lemon juice and autoclaved sugar solutions with those of polyhydric phenols, evidence was obtained that the substance or substances formed from the sugars on autoclaving are most probably related to catechol.

6. On autoclaving decitrated lemon juice or solutions containing the same sugar concentrations a substance is produced which is capable of oxidising *p*-phenylenediamine but not guaiacum or benzidine.

7. Catechol and quinol do not destroy the reducing principle in decitrated lemon juice which has been steamed anaerobically for 1 hour, whilst they do so in the unheated juice.

8. These dihydric phenols are unable to destroy the reducing principle in the absence of an enzymic factor, whilst benzoquinone and the ethereal extract do so unaided.

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CVII. THE HYPERGLYCAEMIA OF CANCER[1].

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An essential difference between tumour tissue and normal tissue is the increased ability of the former to glycolyse carbohydrate. Warburg *et al.* [1923, 1924; Minami, 1923] have shown that isolated but "surviving" carcinoma tissue transforms glucose completely into lactic acid. This sugar cleavage is a source of energy which enables the cancer cell to live anaerobically while under similar conditions the normal cell dies. Depriving the cancer cell of this source of energy results in retarded growth; increasing it, by increasing the concentration of glucose, results in accelerated growth. Cori and Cori [1925] have correlated these observations of Warburg with the glucose metabolism of the cancer cell in the living body. They found that blood, in passing through a tumour *in situ*, gains an amount of lactic acid almost equivalent to the quantity of sugar disappearing. Thus cancer cells *in vivo* also utilise sugar differently from the normal. Fundamental studies such as these emphasise the need for a thorough investigation of the carbohydrate metabolism of the cancer cell both in the isolated tissue and in the living body.

A significant fact in this connection is that the energy which the cancer cell obtains by glycolysis depends upon the concentration of glucose in the medium in which it lives. Cori and Cori [1928] have shown that at a normal blood-sugar level the tumour derives 15 % of its energy from glycolysis. At higher blood-sugar levels there is a greater rate of glycolysis. The experiments of Warburg, Wind and Negelein [1926] on isolated tumour material show that a slight increase over the normal glucose concentration has a marked influence on glycolysis. Doubling the sugar concentration by increasing it from 50 to 100 mg./100 cc. more than doubled the rate of lactic acid formation. This important relationship existing between sugar concentration and cellular activity is further brought out by our observation in collaboration with Hueper [1931] that there is a correlation between the amount of blood-sugar of a cancer patient and the malignancy of the tumour as expressed by the number of mitoses in a histological section. The higher the blood-sugar, the greater was the number of cell divisions.

[1] Reported before the Division of Medicinal Chemistry of the American Chemical Society, Buffalo, N.Y., September 2, 1931.

Numerous investigations carried out on the carbohydrate metabolism of cancer patients have been concerned with sugar tolerance. The results in general show that the blood-sugar level is raised to a point considerably higher in the cancer patient than in a normal individual; that this maximum is reached more slowly; and that the return to the original level is considerably retarded. The figures of Slosse and Reding [1927] show that the average increase in blood-sugar in a cancer patient after ingestion of 50 g. of glucose is 108 mg./100 cc., thus bringing the hyperglycaemia generally to over 200 mg./100 cc. It requires 2 to 3 hours for the original level to be attained again. Thus it is possible that during one-third of the time throughout a day the cancer patient's blood-sugar level is over 150 mg./100 cc. D'Halluin [1930] agrees with Slosse and Reding in that ingestion of 50 g. of glucose always produces, in the cancer patient, an increase greater than 45 mg./100 cc. in the blood-sugar, but his maximum is not as high.

In regard to the fasting blood-sugar level of cancer patients, however, much less information is available nor is there such good agreement. Slosse and Reding's figures, taken from their tolerance curves, show that the fasting blood-sugar level of cancer patients is definitely above normal—if averaged, 18 mg./100 cc.—although they themselves do not draw this conclusion. D'Halluin [1930] states that the fasting blood-sugar level is normal in 75 % of his cases. Chatain [1930] likewise concludes it to be normal.

In view of the disagreement existing on this question, and because of the possible clinical significance attached to it, we undertook to determine the fasting blood-sugar value of a large number of treated and untreated cancer patients. As controls, we also determined the values on a series of normals, a series of benign tumour cases, and a series of medical (non-cancerous and non-diabetic) cases. Our results clearly indicate that the fasting blood-sugar values in untreated cancer cases tend to be high, averaging 20 mg./100 cc. above normal. The values for treated cancer cases and benign tumour cases are also considerably above normal, whereas the medical cases average only slightly above the normal values.

That it has not previously been generally accepted that the fasting blood-sugar value of cancer patients tends to be high appears to be due to two factors.

(1) A distinction has not been made between a real normal value and a high normal.

(2) Most sugar methods, especially those ordinarily used in hospital laboratories, are not specific enough for sugar to bring out small differences.

Blood-sugar values ranging from 70 to 120 mg./100 cc. of blood have generally been accepted as normal values. The blood-sugar value obtained necessarily depends upon the method used in determining it, since none of the blood-sugar methods commonly used is absolutely specific for glucose. Other blood constituents contribute toward the "blood-sugar" value. Using a single method, the range covered can be narrowed. Moreover when a distri-

bution curve is plotted from these figures, the majority of them will be found grouped in a still narrower range. Those values which occur most frequently are the real normal values. Considering the distribution in our normal group

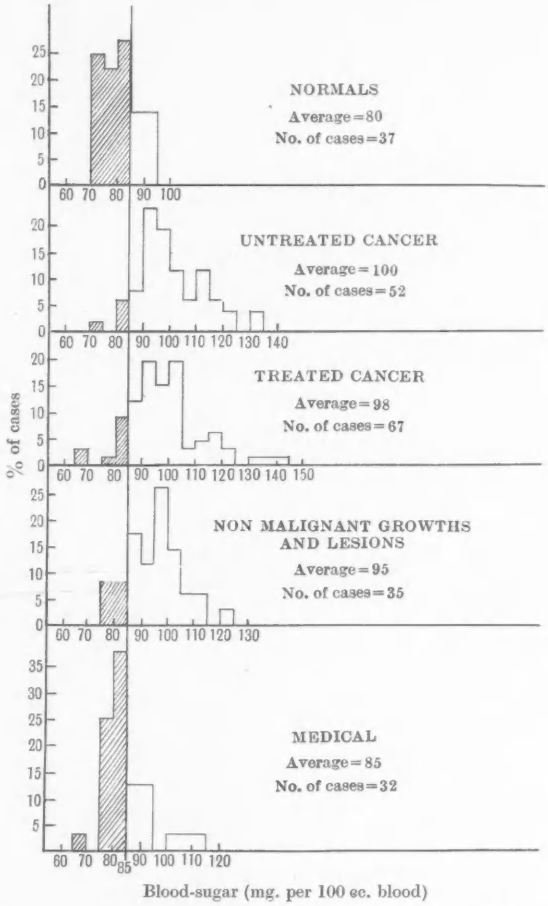

Fig. 1. Blood-sugar determinations. (Shaded area represents values below 85. Plain area represents values above 85.)

(Fig. 1) the majority of the figures (76 %) are found within the range 70 to 85 mg./100 cc. and such will be considered the normal range in discussing the results reported in this paper.

The Hagedorn-Jensen sugar-method.

In the sugar values here reported the Hagedorn-Jensen [1923] sugar-method on oxalated venous blood has been used. The chief reason for adopting this method was that it expresses very nearly the "true" sugar values, being at the same time a micro-method.

In view of the confusion existing as regards the merits of the various copper reagents, these were avoided. Moreover, blood-filtrates of the tungstic or molybdic acid type (Folin-Wu; Benedict) are not suitable for detecting small differences in blood-sugar values. Such filtrates contain, in addition to glucose, many non-sugar reducing substances which act on the reagents used in estimating glucose, and so contribute to the determined value. This non-sugar material has been reported to be very constant in normal as well as in pathological conditions regardless of the blood-sugar level [Somogyi, 1927]. One might suppose, then, that sugar values by any one method would be comparable. If, however, there were a slight variation in this non-sugar fraction, no difference in the final figure would be observed between a blood-filtrate which contained more sugar and less non-sugar reduction and one which contained less sugar but correspondingly more non-sugar reduction. Results which we have obtained on a small number of cancer patients indicate that this non-sugar fraction is less in the blood of cancer patients. Therefore, methods which include amounts of non-sugar reduction in the "blood-sugar" figure could not be expected to bring out slight differences in the "true" sugar values. The Hagedorn-Jensen filtrate is nearly free from these interfering substances and was therefore quite suitable for this purpose, as was also the Somogyi filtrate [1930]. Since our work was started before the latter procedure was available to us it has been used only as a check on some of the results.

Somogyi has recently criticised the Hagedorn-Jensen method by pointing out that the filtrate contains a small amount of non-sugar reducing material which acts on the reagents used for the sugar determination, thus giving a slightly too high sugar value. By modifying the reagents so that the filtrate would be slightly alkaline, he produced a filtrate which contained only traces of non-sugar material. More recently Herbert and Bourne [1930] compared the two methods and found that the Hagedorn-Jensen filtrate gave values on an average only 4 mg. higher than that of Somogyi, hardly outside the experimental error. We have likewise compared the two methods and have found similar results. The agreement was almost always within the limits of experimental error, the Somogyi values in fact averaging 1 mg. higher. Since, according to Somogyi, the complete removal of the non-sugar reducing substances depends upon the alkalinity of the filtrates, we investigated the p_H of our filtrates. Herbert *et al.* [1929, 1930] have reported p_H values of 6·6 and 6·8 for the Hagedorn-Jensen and 7·4 for the Somogyi filtrate. Using Somogyi precipitating reagents which titrated 12·1 cc. of 0·0997 *N* NaOH to

10 cc. of 1·8 % $ZnSO_4$, we found the filtrates to have a p_H of 7·1 to 7·2. In the original Hagedorn-Jensen method the authors did not make a titration between their reagents, but stated that the excess of zinc sulphate was so calculated that the solution was never alkaline. However, the effect of the blood-buffers on this mixture was not taken into consideration. In our experiments the Hagedorn-Jensen and Somogyi zinc sulphate reagents were made from the same stock zinc sulphate (45 %), and the same 0·0997 *N* sodium hydroxide was used. The p_H of Hagedorn-Jensen filtrates prepared with these reagents was 7·0 to 7·1. The similarity in the p_H values of these filtrates may explain the close agreement in the sugar values which we obtained in comparing the two methods. We are able to state that glutathione, believed by Somogyi to be the chief cause of non-sugar reduction, was absent from or negligible in our Hagedorn-Jensen filtrate, for we have added glutathione to plasma in the proportion of 100 mg./100 cc. and practically none (0 to 5 mg. calculated as glucose) was found in the filtrates as an increment over the original sugar figure. With a minimum of non-sugar materials present, it is evident that the results obtained express very closely the true sugar figure unless there is a hitherto unknown reducing substance in cancer blood which comes through in this filtrate.

Kramer and Steiner [1931] have recently criticised the Hagedorn-Jensen method because of errors which occur through the use of cotton in filtration. These authors state that "the technique of filtration is the essential source of inaccuracy which renders the Hagedorn-Jensen method altogether inadequate for analytical work." They claim that 4–22 mg. of glucose are lost by retention in the cotton plug and, moreover, to have confirmed the figures of Folin and Malmros [1929] who found 3–19 mg. lost when 100 mg. of cotton was used. However, the latter pointed out that 100 mg. of cotton were too much; that if only 20 mg. were used there was no error from this cause. At the time we adopted this method for our use two years ago, it seemed that filter-paper would be more convenient. A comparison was made at that time between sugar values obtained on duplicate blood-filtrates, one filtered through exactly 4 mg. of cotton and the other through Whatman No. 1 filter-paper rinsed with distilled water before use. Almost exact agreement was obtained as seen from the following figures:

	Filter-paper mg./100 cc.	Cotton mg./100 cc.
Exp. A (blood sample)	104	106
Exp. B (blood sample)	120	120
(blank)	16	15
	104	105

This is in keeping with Kramer and Steiner's suggestion of the use of filter-paper as an improvement in preparing the Hagedorn-Jensen filtrate.

Further observations on this sugar method have been made as follows.

(1) It is important that the dilute zinc sulphate be fresh. The original authors recommended that this be made from a concentrated stock solution

every 8 days. If the zinc sulphate has deteriorated it produces an increase in the blank value while an increase in the value from the blood-filtrate, if any, is slight. Too high a value being thus obtained in the blank, too low a blood-sugar value will result.

(2) The potassium ferricyanide solution must be checked occasionally. This keeps 6 months at least when stoppered and in the dark; but, if the bottle is in frequent use, a slight deterioration will be noted. When such takes place, it should be discarded and fresh solution taken.

(3) Water which has been kept in the ice-box after being freshly distilled is not necessary. No difference was observed between this and distilled water which had been kept at room temperature for a few days.

(4) It is not necessary to boil the tubes 3 minutes to precipitate the proteins. One minute or until the coagulum rises is sufficient.

Results.

The accompanying diagram, Fig. 1, combines the results which we have obtained in determining the fasting blood-sugar of 37 normals, 52 untreated cancer cases, 67 treated cancer cases, 35 cases of non-malignant growths (untreated) and 32 medical cases.

The normal values cover the range 70–95 mg. of glucose per 100 cc. of blood; but, as discussed previously, owing to the distribution we may consider values between 70 and 85 as the normal values, 76 % of these being within this range. Since such a large proportion of the group is under the figure of 85, we will consider values between 85 and 95 as high normals. The average blood-sugar of the entire group is 80.

The group of untreated cancer cases comprises those cases who had received no medical treatment for the cancerous condition before our determination of their blood-sugar. Diagnosis of malignancy was later confirmed histologically. Any case diagnosed by the physician as also being diabetic was omitted from this and the following groups. None of the cases included showed glycosuria under a normal diet, nor were there any other symptoms of diabetes. An observed hyperglycaemia was, therefore, not due to diabetes mellitus. This group, the untreated cancer cases, shows the highest values for blood-sugar, 90 % having a figure higher than 85, in contrast to the normals, 76 % of which were under this figure. From the graph it is at once apparent that there is a decided shift to the right. The blood-sugars range from 74 to 135 with the peak at 90–100. The average for the group is 20 mg./100 cc. higher than that of the normal group.

By treated cancer cases are meant those who have received one or more X-ray or radium treatments previous to our observation of their blood-sugar. As a group, these cases show almost as high blood-sugar values as the untreated cases. The average value is 98, with the range covered, 66–142. Treatment has not, on the whole, lowered the blood-sugar of this group, although it has done so in certain individual cases.

The non-malignant group includes cases of benign tumours and chronic lesions, all of which are abnormal growths and many of which may be considered precancerous. These cases also have a tendency toward a higher sugar than normal; range, 75–123; average, 95; not, however, quite as high as in the cancer cases.

In contrast to the three latter groups, purely medical cases (diabetes being excluded) do not, in general, show this hyperglycaemic disturbance. The sugar level is quite normal; range, 66–115; average, 85. Since a high blood-sugar is not found in most of these purely medical cases, it is evident that the hyperglycaemia is a characteristic of malignant or precancerous conditions.

Discussion.

This increase in the fasting blood-sugar level, as well as the increased tolerance curve, is in our opinion an expression of the hyperglycaemia of cancer, and since the fasting value is generally determined on hospital patients, it seems worth while to point out its significance. While sugar tolerance curves may give a more complete picture of the ability to metabolise carbohydrate, still the fasting blood-sugar gives a very valuable criterion and is much less difficult to obtain than a complete tolerance test. Since the fasting blood-sugar determination is usually a routine procedure in hospitals and clinics, the value is readily available to the physician. Hitherto, the physician has not been concerned when values of 100 were obtained in cancer patients (100 by the Hagedorn-Jensen method corresponding to about 120 by the Folin-Wu method which is generally used in hospital laboratories). Our results show that such a figure is about 15 mg./100 cc. higher than normal.

It should not be concluded from these results that a high blood-sugar is of any value in the diagnosis of cancer patients because it is not a constant phenomenon. Its importance lies in the prognosis and treatment of the patient. Patients with a high blood-sugar, as Jackson [1929] has pointed out, have a poor prognosis; those with a low blood-sugar respond to almost any form of treatment.

In view of the effect of sugar concentration on the activity of tumours, Cori and Cori [1928] have discussed the question as to whether patients suffering from malignancy would derive any benefit from a low-carbohydrate diet. Their conclusion was that, since the hyperglycaemia following an ordinary meal rarely exceeded 150 mg./100 cc., a severe restriction of carbohydrate seemed unnecessary, but that food which tended to raise the blood-sugar above this level should be avoided. However, as we have pointed out, it is quite probable that in the cancer patient the hyperglycaemia is greater than 150 mg./100 cc. during a considerable part of the day. This fact becomes still more significant when considered in the light of our findings that even the fasting blood-sugar values tend to be considerably above normal.

In our experience, the clinical condition of the patient seems to run

parallel with the variations of the fasting blood-sugar level. If any type of treatment benefits him, the hyperglycaemic level is reduced; if the clinical condition is aggravated, the blood-sugar level is increased. Reduction of the blood-sugar level should, therefore, be beneficial to the patient. Reding (private communication) and Jackson have both reported this to be the case. In any event, blood-sugar values should be carefully watched in connection with treatment in order that the clinical condition may not be aggravated.

On the other hand, does lowering of the blood-sugar in a cancer patient make him respond to that particular type of treatment which aggravates his condition at the higher blood-sugar level? This phase of the problem is being investigated in our laboratories. Thorough and well-controlled clinical observations will be needed to exclude the possibility of any untoward effects before therapeutic measures tending to lower the blood-sugar can be recommended as a supporting therapy.

The fact that the treated cancer cases as a group show practically as high sugar values as untreated cases, may mean that treatment has had no effect on the blood-sugar, or it may mean that while in some of the cases the blood-sugar has been lowered in a corresponding number it has been increased. This indicates the importance, however, of carefully observing the effect of treatment on the blood-sugar of the individual patient.

Summary.

1. The average blood-sugar level in 52 untreated cancer cases, as determined by the Hagedorn-Jensen method, is 20 mg./100 cc. above normal.

2. Treated cancer cases and cases of non-malignant (benign) growths also show blood-sugar values higher than normal, the treated cases being, on the whole, almost as high as the untreated.

3. Purely medical cases do not, in general, show an increase in the blood-sugar level.

4. Relations between blood-sugar, prognosis and treatment are discussed.

Our appreciation is due to Dr E. F. Schroeder of this laboratory for his advice.

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CVIII. CHOLEIC ACIDS.

IV. KETO-ENOL TAUTOMERISM AND CO-ORDINATION COMPOUNDS.

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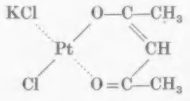
An important isomerism occurring in substances of biochemical interest is the keto-enol tautomerism. The peculiarities of the configurations

$$R_1.CO.CH_2.R_2 \text{ keto} \rightleftharpoons \text{enol } R_1.C(OH):CH.R_2,$$

the state of equilibrium between both forms under varying conditions, and the rates of their interconversion have been elucidated by Meyer and co-workers [1911–20] in the case of ethyl acetoacetate and of some other substances of similar configuration. While Meyer and others have recognised and studied the position of the equilibrium in relation to the substituents R_1 and R_2, and also the influence of temperature, solvent and acidity on the keto-enol equilibrium [Meyer and Kappelmeier, 1911; Meyer, 1912], nothing is known in regard to keto-enol equilibrium in co-ordination compounds.

Substances containing reactive methylene groups are endowed with unsaturated partial valencies and are, therefore, apt to enter into co-ordination compounds. Werner [1901] was the first to describe complex compounds of acetylacetone like



Many related compounds with various metallic salts have since been prepared, *e.g.* $CH_3COCH_2COCH_3.AlBr_3$ by Kohler [1902], $C_6H_5COCH_2COCH_3.SbCl_5$ from benzoylacetone, $CH_3COCH_2COOC_2H_5.HSbCl_6$ and $CH_3COCH_2COOC_2H_5.SbCl_5.2HSbCl_6$ from ethyl acetoacetate by Rosenheim *et al.* [1903], the last containing 3 metal atoms for 1 molecule ethyl acetoacetate [*cf.* Pfeiffer, 1927]. The tautomeric substance has to be formulated as enol in the majority of these mixed inorganic-organic molecular compounds, and the enol hydrogen is replaced by one main valence of the metal. For other compounds, no decision regarding tautomerism was sought and we might attribute either configuration to purely organic co-ordination compounds like the compounds of ethyl acetoacetate with acid potassium trichloroacetate and with benzidine, $CH_3COCH_2COOC_2H_5$.

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$(CCl_3COO)_2KH$ [Koloseff, 1903] and $CH_3COCH_2COOC_2H_5.2NH_2.C_6H_4.C_6H_4.NH_2$ [Heidrich, 1898].

While it is hardly possible to apply the bromine titration method of Meyer to these compounds, the co-ordination compounds of tautomeric substances with bile acids offer an opportunity for such a study. With this object in view, we prepared the choleic acids of ethyl acetoacetate, acetylacetone and benzoylacetone. These compounds are easily obtained by dissolving both the tautomeric substance and the desoxycholic acid in absolute alcohol.

The analysis of the diketone-choleic acids offered some difficulties, as the enol forms behave like weak acids and thus interfere with the estimation of the acid equivalents of the choleic acids. The alcoholic solutions of these diketones react acid to varying degrees, but their hydrogen ion dissociation and its relation to tautomerism has never been studied in a quantitative manner. Since no sharp end-point could be ascertained with bromothymol blue, a more acid indicator was employed in the determination of their acid equivalent. As desoxycholic acid on the other hand is not a very strong acid there was a limit on the acid side for the choice of the indicator. The most satisfactory end-point was obtained with bromocresol purple and this was used for benzoylacetone-choleic acid.

No consistent figures could be obtained for acetylacetone-choleic acid, because the enolised diketone will neutralise considerable amounts of alkali before the bile acid is completely neutralised. Neither could this choleic acid be titrated like the choleic acid of a fairly strong acid, as the enolisation of the keto-portion does not take place instantaneously. On the other hand, the back-titration with acid after having added an excess of alkali is not feasible, as there is no sharp demarcation between the dissociation of the first and second enol groups of this diketone.

The acid equivalents suggest a co-ordination number 3 for ethyl acetoacetate and for benzoylacetone. In order to corroborate this result we resorted to other analytical procedures. Ethyl acetoacetate on treatment with sulphuric acid decomposes to acetone and carbon dioxide. The acetone formed according to this reaction from ethyl acetoacetate-choleic acid was determined by the method of Engfeldt [1917]. This analysis conformed with the assumption of a tricholeic acid. Another sample of ethyl acetoacetate-choleic acid was subjected to repeated steam-distillations under reduced pressure until the non-volatile residue attained constant weight. The loss of volatile matter tallied with the percentage of ethyl acetoacetate calculated for a tricholeic acid.

The enol titration of these choleic acids yielded striking results. When they were dissolved rapidly in cold alcohol and treated with alcoholic bromine solution, the following enol percentages were obtained: 8·4 % for ethyl acetoacetate-choleic acid, 11·95 % for benzoylacetone-choleic acid and 7·75–7·9 % for acetylacetone-choleic acid. The computed percentages of acholic component for tricholeic acids are in the order given, 9·95 %, 12·1 % and 7·85 %. A comparison of these two sets of figures permits the following conclusions. (1) These compounds, including the one of acetylacetone for which no other analytical evidence was accessible, contain three molecules desoxycholic acid for one molecule of the tautomeric substance, (2) The tautomeric constituent is bound in completely enolised state.

In alcoholic solution more than 99 % of benzoylacetone is present in the enol form. Thus, the complete enolisation of this substance in molecular

combination with a bile acid is not surprising and may serve as a control. Acetylacetone in the liquid state is a mixture of 80 % enol and 20 % ketone, while the enol form is increased to 84 % after a solution in absolute alcohol has attained equilibrium. We found 76 % enol after prolonged standing in 95 % alcohol. The figures for freshly dissolved acetylacetone-choleic acid are distinctly higher; they range between 91 and 101 %, and support the assumption that acetylacetone is completely enolised in its co-ordination compound.

Ethyl acetoacetate contains 7·4 % enol in undiluted form, 12 % in absolute alcohol, 8·7 % in 96 % alcohol and 8·0 % in 95 % alcohol. In sharp contrast to these data, ethyl acetoacetate-choleic acid contained as much as 84·6 % enol at the time when bromine was added. This figure and its rapid decrease within a few minutes implies that this choleic acid is likewise completely enolised, although its original enol share of 100 % changes so rapidly as to escape experimental verification. It is noteworthy in this connection that the benzoylacetone-choleic acid is completely precipitated to the point of solidification immediately upon mixture of concentrated alcoholic solutions of its constituents. The acetylacetone-choleic acid is formed at a slower rate, but after 5–10 minutes the concentrated alcoholic solution likewise solidifies. The ethyl acetoacetate-choleic acid crystallises overnight. Thus, the rate of formation of these choleic acids increases with the enol content of the free tautomeric component.

If the solutions of these choleic acids are allowed to stand for prolonged intervals before determination of the enol, considerable ketonisation takes place in the case of acetylacetone and ethyl acetoacetate. The final enol percentage is about 13 % for the ester-choleic acid in 95 % alcohol, and 15 % in absolute alcohol, while the enol content of acetylacetone-choleic acid drops to about 70 %. These figures differ slightly from the keto-enol equilibrium of the free substances, but they represent true equilibria, as an equal enol percentage is reached in a solution resulting from combination of alcoholic solution of ethyl acetoacetate and of desoxycholic acid.

These observations may be interpreted in regard to the molecular dissociation of choleic acids in solution. Unpublished experiments on the molecular weight of choleic acids of simple aliphatic acids favour the assumption that choleic acids, when dissolved in alcohol, completely dissociate into their molecular components. Thus, we are disinclined to attribute the difference in the final equilibrium between solutions of free and of combined ethyl acetoacetate to the existence of choleic acid in solution. Molecular dissociation of these choleic acids is practically instantaneous. Repeated experiments demonstrated beyond doubt that very rapid manipulation leads to lower enol figures than the maximal figures given above. This observation applies to all three substances used. When less than 30 seconds elapsed between solution of the choleic acid and the addition of the bromine solution, enol values of 73·8, 91 and 75 % were obtained for ethyl acetoacetate-, acetylacetone-, and benzoylacetone-choleic acid respectively. The deviation from 100 % appears

to be due to the presence of undissociated choleic acid, in which one can imagine the double bond of the enol group screened from the bromine by the surrounding bile acid molecules. Studies on the kinetics of tautomerisation of these substances are reported elsewhere [Sobotka and Kahn, 1932].

Another case of tautomerism which we studied in this connection was that of anthranol (9-hydroxyanthracene) and anthrone (9-ketodihydroanthracene) [Meyer, 1910]. These substances do not tautomerise as readily as ethyl acetoacetate and the aliphatic diketones. They have been isolated separately. The ketone is a white substance, while the enol is deep yellow and displays a brilliant blue fluorescence in solution. The disappearance of this fluorescence serves as indicator in the titration of anthranol-anthrone mixtures with dilute alcoholic bromine solution [Meyer and Sander, 1912–13].

We synthesised a choleic acid from an alcoholic solution of anthrone which had reached equilibrium at about 15 % enol. The crystals obtained were almost white and showed no fluorescence initially when dissolved in alcohol. After 1½ hours, the solution had developed a strong blue fluorescence and the enol content was 14 % according to a bromine titration. This enolisation could be hastened by addition of a trace of hydrochloric acid and by heating. It follows from these observations, that in this instance the choleic acid contains the keto-form of the desmotropic pair.

Tautomerism is one instance in which the presence of a substance in two forms of varying reactivity can be demonstrated by analysis and preparative isolation. But there are many other substances in which the rearrangement into a more reactive form is conjectured without direct proof. It is a function of catalysts to elicit or aid such rearrangements. The present experiments exemplify how a substance may be converted into a very reactive form by the rather gentle reaction of molecular combination with bile acid. This might serve as an example of one of the many functions of bile acids *in vivo*. The demonstration of the enolising influence exercised by bile acids on free acetoacetic acid and other β-keto-acids offers experimental difficulties. It is plausible however to attribute to the bile acids a function in the intrahepatic β-oxidation of fatty acids through enolisation of the intermediary keto-acids.

Experimental part.

Benzoylacetone-choleic acid was prepared by mixing a solution of 1·5 g. of benzoylacetone of M.P. 61° in 10 cc. absolute alcohol with a solution of 10 g. desoxycholic acid in 25 cc. alcohol. The choleic acid precipitated and the solution solidified. The substance was recrystallised from alcohol and melted at 185° (corr.). 88·4 mg. and 92·4 mg. dissolved in alcohol titrated in the presence of bromocresol purple required 3·96 cc. and 4·36 cc. 0·05 *N* NaOH. The acid equivalent is therefore 446–447, while the acid equivalents calculated for di-, tri- and tetra-choleic acids are 473, 446 and 433 respectively. (The bromine titrations are reported under a separate heading.)

Ethyl acetoacetate-choleic acid was prepared in an analogous manner. The crystals were colourless and melted at 154° (corr.). 203·5 mg. in ethyl alcohol required 4·58 cc. 0·05 *N* NaOH with bromocresol purple, and 4·68 cc. with bromothymol blue. The acid equivalents calculated from these figures are 444·5 and 435. The theoretical value for tricholeic acid is 435·6. In addition to this analysis, 256·4 mg. ethyl acetoacetate-choleic acid were digested with 10 cc. 20 % sulphuric acid for 10 hours on the water-bath. The solution was then treated according to the method of Engfeldt [1917]. 10·75 cc. 0·1 *N* iodine used corresponded to 10·4 mg. acetone or 23·1 mg. ethyl acetoacetate, while the calculated values were 11·3 mg. acetone or 25·4 mg. ester.

The desoxycholic acid contained in this choleic acid was determined as follows. 518·8 mg. choleic acid were placed with 10 cc. water in a small double-neck distilling flask which had been weighed with a capillary tube and rubber stopper. The water was distilled off under reduced pressure, it was then replaced three times and each time removed to dryness until the distillate failed to give the purple reaction with $FeCl_3$ characteristic for ethyl acetoacetate. The flask had lost 52·0 mg. or 10·02 % of the weighed amount of choleic acid. Its theoretical percentage of ethyl acetoacetate is 9·95 %.

Acetylacetone-choleic acid was prepared by adding 1·5 g. of the diketone to 11 g. desoxycholic acid in 30 cc. absolute alcohol. The yield was 5·7 g. of crystals melting at 162°.

Enol titration. The titration of the enol was carried out by dissolving a weighed amount of substance in chilled alcohol. After the time given in Table I had elapsed, 15–25 cc. of an approximately 0·2 *N* alcoholic solution of bromine was added rapidly. The reaction of the bromine with the unsaturated compound takes place at once and the excess bromine is removed by addition of 2 cc. of a 10 % alcoholic solution of β-naphthol which instantly decolorises the mixture. 1 cc. of glacial acetic acid and about 5 cc. of an aqueous 20 % solution of potassium iodide cause the liberation of free iodine and the completion of the reaction is insured by placing the Erlenmeyer flask for 10 minutes on the water-bath. The iodine is then titrated with 0·1 *N* thiosulphate without indicator. Another 10 minutes on the water-bath will bring out an additional 0·1 to 0·3 cc. 0·1 *N* iodine. The amount of enol equivalent to 1 cc. 0·1 *N* thiosulphate, is calculated from

$$\frac{CH_3.C(OH):CH.COOC_2H_5}{2 \times 10}$$

or 65 mg., for acetylacetone 50 mg., and for benzoylacetone 81 mg.

The results of these titrations are summarised in Table I. All titrations were carried out in 95 % alcohol.

Anthrone-choleic acid was prepared by dissolving 3 g. desoxycholic acid in a solution of 0·5 g. anthrone in 10 cc. alcohol. Upon standing, pale crystals of M.P. 179° separated. Their acid equivalent was 441. The theoretical acid equivalent for an anthrone-tetracholeic acid is 437·8.

Table I. *Enol titration of choleic acids with a tautomeric component.*

Substance	Time elapsed between solution and addition of bromine	Amount of substance (mg.)	0·1 *N* thio-sulphate (cc.)	Enol form (mg.)	Enol form % of choleic acid	Enol form % of tautomeric component
Benzoylacetone-, choleic acid (0°)	10 secs.	505	5·70	46·2	9·1	75
	10 secs.	491	6·00	48·6	9·9	82
	1 min.	621	9·15	74·2	11·95	99
Acetylacetone-choleic acid (0°)	10 secs.	453	6·25	31·3	7·25	91
	1 min.	403	6·23	31·2	7·75	99
	2 mins.	408	6·45	32·3	7·9	100
	1 hr.	298	4·32	21·6	7·25	91
	48 hrs.	447	4·75	23·7	5·3	69
Ethyl acetoacetate-choleic acid (0°)	10 secs.	358·5	5·43	26·3	7·34	73·8
	20 secs.	563	6·65	43·2	7·68	77·2
	30 secs.	299	3·87	25·2	8·43	84·7
	30 secs.	298	3·72	24·2	8·12	81·6
	2 mins.	472	5·06	32·9	6·97	70·0
	4 mins.	472	4·13	26·8	5·68	57·1
	11 mins.	536	1·97	12·8	2·39	24·0
	48 hrs.	472	1·00	6·5	1·38	13·8
Ethyl acetoacetate-choleic acid (−10°)	2 mins.	308·5	3·80	24·7	8·01	80·5
	30 mins.	308·5	3·30	21·4	6·94	69·7
	75 mins.	308·5	2·90	18·9	6·13	61·6
	17 hrs.	308·5	0·65	4·2	1·36	13·6
Ethyl acetoacetate *plus* desoxycholic acid (0°)	17 hrs.	500 + 5000	10·45	68·0	—	13·6
Desoxycholic acid (0°)		500	<0·10	—	—	—

Summary.

The preparation and analysis of co-ordination compounds consisting of three molecules desoxycholic acid and one molecule of a tautomeric substance are described. While these substances in the free state contain varying proportions of keto-form, they are completely enolised when bound in choleic acids. However, anthrone-tetracholeic acid contains the acholic component in the keto-form.

There is evidence of rapid and complete molecular dissociation when these choleic acids are dissolved. The bearing of these observations on the mechanism of β-oxidation is discussed.

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Addendum (*June 29th, 1932*). Our conceptions dovetail perfectly with a hypothesis which Fowweather reached from quite a different source [*Biochem. J.* 1932, **26**, 165]. He suggests that tautomerism may account for the difference between bilirubin preparations giving a direct van den Berg reaction and those giving only an indirect one. Fowweather adduces evidence that promptly diazotisable bilirubin, as obtained from bile, is the enol form and he concludes that some bile constituent, presumably bile acid, enolises the pigment and has a stabilising influence on its enol form.

CIX. CHOLEIC ACIDS.

V. THE SEPARATION OF OPTICAL ANTIPODES BY MEANS OF CO-ORDINATION COMPOUNDS[1].

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Of the three methods for the separation of optical antipodes from racemic mixtures originated by Pasteur, the mechanical selection of crystals of asymmetrical habit, the formation of chemical compounds, as salts of a racemic acid with an optically active base, and the destruction of one moiety of the racemate by micro-organisms, the second was used by Marckwald and McKenzie [1899, *cf.* Marckwald and Meth, 1905] for resolution of optically active alcohols through their esters. The third method has been extended by the use of extracellular enzymes instead of micro-organisms and Bredig and Fajans [1909] devised a model for asymmetric catalysis on a purely chemical basis. Willstätter [1904] attempted to separate racemic alkaloids by "dyeing" them on wool. The process of dyeing was explained as a salt formation between the dye and the optically active protein of the animal fibre and thus the experiment would fall under Pasteur's second method. Accepting theories which explain dyeing by co-ordinative valencies, we would classify Willstätter's experiment with the present attempts to resolve racemates by means of co-ordination compounds. Successful experiments with optically active dyes have since been reported by Porter and Ihrig [1919, 1923], and Ingersoll and Adams [1922], while Fischgold and Ammon [1931] obtained evidence of optically selective adsorption of mandelic acid in presence of alkaloids on optically inactive adsorbents.

An important step in this direction was made by Windaus *et al.* [1922–23] who studied the addition compounds of digitonin with such alcohols as α-terpineol, *ac*-tetrahydro-β-naphthol or carvomenthol and observed a great difference in solubility between the *d*-alcohol-digitonide and the *l*-alcohol-digitonide. The utilisation of this property enabled them to effect an almost complete resolution of *dl*-α-terpineol and *dl-ac*-tetrahydro-β-naphthol into active components.

In the course of our studies on choleic acids, we tried the synthesis of addition compounds of desoxycholic acid with racemates representing various types of compounds, such as hydrocarbons, alcohols, ketones and acids.

[1] A report on these experiments was read before the Division of Organic Chemistry at the Annual Meeting of the American Chemical Society at Buffalo, September 2, 1931.

A preliminary report on these experiments was given in 1931[1]. Methylethylacetic acid was chosen as the simplest aliphatic monocarboxylic acid with an asymmetrical carbon atom. Methylethylacetic acid-choleic acid obtained by direct synthesis of its constituents contained an excess of $\frac{1}{6}$ of the laevo- over the dextro-form of the fatty acid. No choleic acid could be obtained from mandelic acid. Smaller yet definite preference in the formation of co-ordination compounds was given by the laevorotatory forms of phenylethylethanol and dipentene. Benzoin would not form a choleic acid co-ordination compound under the conditions of our experiments. Two choleic acids, containing one and two molecules of bile acid for each molecule of the ketone, have been described by Rheinboldt *et al.* [1929] for natural *d*-camphor. In the case of synthetic *dl*-camphor a dicholeic acid was obtained, which contained an excess of $\frac{1}{8}$ of the laevorotatory camphor. The laevo-form gives a choleic acid less soluble and presumably more stable than its enantiomorph. In summarising these experiments, one observes that the bile acid, which is dextrorotatory, prefers the laevo-form of each pair. Digitonin, a laevorotatory substance, formed more insoluble compounds with *l*-α-terpineol and *l*-actetrahydro-β-naphthol [Windaus *et al.*, 1922–23]. Thus, no rule for the formation of co-ordination compounds can be based on the sense of rotation of the components. However, the difference in behaviour of optical antipodes towards natural products like bile acids or saponins helps in classifying one of a pair as the physiological enantiomorph.

While this method is not intended to replace the traditional methods of resolution in their established sphere, it widens the range of resolvable racemates. A study of the most suitable concentrations and solvents will without doubt guide us towards a practical procedure in the preparative employment of this principle, which applies to a great variety of substances. They comprise many groups of relatively inert substances unfit for salt or ester formation, *e.g.* hydrocarbons. But apart from its preparative merits, this method will aid in the decision whether the incompletely known structure of some inactive substance contains an asymmetric centre or not.

Bile salts are known to influence the action of lipolytic enzymes. Thus the optical selectivity of bile acids may play a part in the optical selectivity of esterases and lipases, a phenomenon discovered by Dakin [1903, 1904, 1905, *cf.* Fischer and Bergell, 1903, Kuhn, 1925]. Moreover, the modern theory of enzyme action has advanced to a point where certain groups of the substrates are recognised as responsible for the affinity between enzyme and substrate [Balls, 1930; Waldschmidt-Leitz, 1931]. The optical selectivity of bile acids, together with some of their other features such as structural selectivity [Sobotka and Goldberg, 1932, Part III] and affinity for certain indicators, suggests them as models for the mode of attachment of lipolytic enzymes to lipoid substances.

[1] Since this publication, Weiss and Abeles [1931–32] reported the isolation of *l-sec.*butyl picramide from *dl-sec.*butyl picramide by formation of a co-ordination compound with *d*-β-naphthylcamphylamine.

Experimental part.

Methylethylacetic acid-choleic acid. 5 g. purest desoxycholic acid, prepared according to Sobotka and Goldberg [1932, Part II], were dissolved in 20 cc. pure inactive methylethylacetic acid and kept in the ice-chest for 24 hours. A crop of 5 g. of crystals was separated. The dried substance showed an acid equivalent of 294–297, while 295·5 is the theoretical value for a dicholeic acid of a valeric acid. The crystals were digested with hot benzene and the benzene treatment repeated two or three times. The combined filtrates were distilled and left a small yellowish glassy residue of bile acid. The fraction of the distillate consisting of methylethylacetic acid was dissolved in the receiving flask with absolute alcohol and made up to 5 cc. It showed $\alpha_D = -0{\cdot}09°$ in a 2 dm. tube. In another experiment with similar proportions, $-0{\cdot}10°$ was the rotation of this fraction.

It was possible to recover a greater excess of *d*-methylethylacetic acid from the mother-liquor than of *l*-acid from the choleic acid. The filtrate, after removal of the crystals in the above experiment, was distilled. The distillate had $\alpha_D = +0{\cdot}2°$ at 27° in a 2 dm. tube. This corresponds to an excess of 0·58 % *d*-methylethylacetic acid of $[\alpha]_D = +17{\cdot}5°$ or 93 mg. for the total distillate of 16 cc. As 5 g. methylethylacetic-dicholeic acid contains 575 mg. methylethylacetic acid, the excess of *d*-form was $\frac{1}{6}$ of the amount that could be left over, if the desoxycholic acid combined with the *l*-form exclusively.

Phenylethylethanol-choleic acid. 7 g. pure desoxycholic acid were dissolved in 20 g. phenylethylethanol $(C_6H_5)(C_2H_5)CH.CH_2OH$. Transparent crystals appeared on slow cooling. They were separated from the mother-liquor and decomposed with benzene. The benzene solution, separated from the insoluble benzene-choleic acid, was expected to contain the phenylethylethanol that had combined with the bile acid. It was distilled under reduced pressure leaving behind small amounts of bile acid that had passed into the benzene solution. The higher boiling fraction of the distillate, made up to 5 cc. in absolute alcohol, showed a rotation of $-0{\cdot}20°$ in a 2 dm. tube. Assuming the $[\alpha]_D$ of *l*-phenylethylethanol to be 37·6° in absolute alcohol [Levene, Mikeska and Passoth, 1930], this rotation would signify an excess of 13·5 mg. *l*-form in the entire amount of phenylethylethanol recovered from 6 g. choleic acid. The undiluted mother-liquor, freed from dissolved bile acid by vacuum distillation, showed slight dextrorotation ($+0{\cdot}05°$, $l = 2$ dm.) corresponding to a concentration of 0·14 % excess *d*-form, the $[\alpha]_D$ of phenylethylethanol without solvent being 18·9°. This excess corresponds to 21 mg. for a total of 15 cc. of mother-liquor recovered.

Dipentene-choleic acid. 6 cc. of dipentene of $n_D^{20} = 1{\cdot}4750$, prepared from terpin hydrate, by boiling it for 10 hours with three equivalents of acetic anhydride according to Ginsberg [1897], were dissolved in 110 cc. hot absolute alcohol containing 24 g. desoxycholic acid. 11·4 g. of crystallised dipentene-choleic acid was obtained on cooling, M.P. 192° (corr.).

The probable co-ordination number is 3 as the acid equivalent was 444,

and the theoretical value for a tricholeic acid $C_{10}H_{16}.3C_{24}H_{40}O_4$ is 438, while $C_{10}H_{16}.2C_{24}H_{40}O_4$ demands 460.

In this instance, dissociation of the choleic acid was accomplished by treating the 11 g. of dried crystals with 25 cc. *N* NaOH and extracting the hydrocarbon with ether. The high-boiling fraction of the ethereal extract, made up to 5 cc. with absolute alcohol, had $\alpha_D = -0{\cdot}08°$ in a 2 dm. tube. This means an excess of *l*-limonene over *d*-limonene of $1\frac{2}{3}$ mg. as the specific rotation of limonene in alcohol is + 120°. The mother-liquor was evaporated to remove the alcohol, the residue was dissolved in benzene, filtered, and the filtrate distilled. The dipentene fraction of about 4 cc. volume was made up to 5 cc. and had $\alpha_D = +0{\cdot}09°$ ($l = 2$ dm.). While the rotation matches the rotation observed in the opposite sense with the limonene liberated from its choleic acid, both are exceedingly small in consideration of the high rotation of the limonene antipodes.

Camphor-choleic acid. More encouraging results than in the case of dipentene were obtained with camphor. 2 g. synthetic *dl*-camphor which showed practically no rotation (less than $-0{\cdot}05°$ in 20 % solution, 2 dm., $[\alpha]_D$ less than $-0{\cdot}12°$) and 7 g. desoxycholic acid were dissolved in 12 cc. hot alcohol. The white crystals of camphor-choleic acid, obtained on cooling, melted at 183° (corr.), as does *d*-camphor-choleic acid. The acid equivalent was 478 while the theory requires 468·4 for a camphor-dicholeic acid. Recrystallisation from ethyl alcohol yielded 2·8 g. substance of the same melting-point and acid equivalent 475. It was decomposed by means of hot benzene. 193 mg. of camphor were isolated having $\alpha_D = -0{\cdot}42°$ in 5 cc. in a 2 dm. tube

$$[\alpha]_D^{23°} = \frac{-0{\cdot}42° \times 5}{2 \times 0{\cdot}193} = -5{\cdot}44° \text{ (absolute alcohol).}$$

Thus the excess of *l*-camphor in the choleic acid was at least $\frac{1}{8}$ of the amount theoretically possible. As 2·8 g. of camphor dicholeic acid contain 446 mg. camphor, there must have been 195 mg. of *d*-camphor and 251 mg. of *l*-camphor.

590 mg. camphor could be isolated by sublimation from the combined benzene filtrates derived from the original mother-liquor

$$[\alpha]_D^{23°} \text{ in alcohol } \frac{+0{\cdot}41° \times 5}{2 \times 0{\cdot}590} = +1{\cdot}74°.$$

The excess of *d*-camphor was about 4 % of the camphor remaining in the mother-liquor.

Summary.

The choleic acid principle can be utilised for the resolution of racemates. Examples of this procedure are given and its bearing on preparative and enzymic problems is discussed.

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CX. INFLUENCE OF GUANIDINE AND ITS DERIVATIVES ON THE ACTION OF AMYLASE.

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Sherman [1923] and other authors have found that the hydrolysis of starch is accelerated in the presence of amino-acids. Filipowicz [1931] has found that neither the amino-group separately nor the carboxyl group of amino-acids possesses any accelerating influence in this reaction. In the present paper the effect of adding guanidine or its derivatives to the systems amylase-starch or amylase-glycogen are described.

Experimental.

The polysaccharides taken were Merck's soluble starch, and glycogen prepared from rabbit-livers. The enzyme was either Merck's "diastase abs." or salivary ptyalin. The p_H of the solutions was adjusted by addition of Sørensen's phosphate buffers, p_H being determined potentiometrically or colorimetrically by means of Clark and Lubs's indicators. Reducing sugars were determined by Bertrand's method, and residual glycogen was determined nephelometrically according to Rona and van Eweyk [1924].

1. *Guanidine.*

A number of systems were set up, containing 30 cc. of 1 % starch solution, 40 cc. of Sørensen's phosphate buffers (p_H 6·1), guanidine carbonate solution from 0 to 0·2 %, and water to 90 cc. The flasks were left for 20 mins. at 34° after which 10 cc. of 0·1 % amylase solution were added to all flasks, except the control to which 10 cc. of water were added. Reducing sugar was determined in 20 cc. portions taken from all flasks at 10 min. intervals after the addition of amylase. The maximum yield of maltose calculated from the glucose content of 20 cc. of the control solutions after hydrolysis with HCl amounted to 60 mg., so that the percentage hydrolysis as given in Table I represents a value of about 57 % after 90 mins.

It follows from Table I that guanidine retards reaction to an extent increasing with its concentration, and that percentage retardation diminishes with time.

Table I. *Effect of varying concentration of guanidine.*

Conc. of guanidine carbonate %	20 mins.		30 mins.		60 mins.	
	Maltose mg.	Retardation %	Maltose mg.	Retardation %	Maltose mg.	Retardation %
0	19·3	—	26·0	—	38·9	—
0·04	18·2	5·7	24·5	5·8	37·0	4·9
0·06	17·6	8·8	24·0	7·7	37·0	4·9
0·08	17·6	8·8	24·0	7·7	37·0	4·9
0·10	14·8	23·3	22·2	14·6	35·4	8·9
0·12	14·8	23·3	22·2	14·6	35·4	8·9
0·14	14·2	26·4	22·2	14·6	35·4	8·9
0·16	14·2	26·4	20·5	21·2	34·2	12·1
0·18	12·5	35·2	20·5	21·2	34·2	12·1
0·20	10·8	44·0	17·6	32·3	34·2	12·1

2. *Action of guanidine in systems containing glycogen and ptyalin.*

Substantially the same results are obtained if glycogen is substituted for starch or ptyalin for Merck's amylase. The conditions of the experiments as well as the compositions of the systems were the same as in the preceding experiments. In systems (p_H 6·1) in which the concentration of starch or glycogen was 0·4 %, of guanidine carbonate 0·1 %, and of Merck's amylase 0·08 %, the retardation of reaction amounted to 19·2 % for starch and 21·8 % for glycogen, after keeping for 1 hr. at 34°. Under similar conditions, but taking saliva (0·02 %) in place of Merck's amylase, the corresponding retardations of the reaction were 25·8 and 26·3 %.

This result was confirmed by experiments in which residual glycogen was determined nephelometrically and the maltose produced was determined by Bertrand's method. The systems consisted of 30 cc. of 1 % glycogen, 80 cc. of Sørensen's buffers (p_H 6·1), 20 cc. of water or 0·9 % guanidine carbonate, and 20 cc. of 0·1 % amylase, added to the preceding solutions after they had been warmed to 34°. Portions of the systems were taken at 10–20 min. intervals for determination of reducing sugars and glycogen. The results (Table II) indicate that the process of hydrolysis of glycogen proceeds parallel with that of production of reducing sugars, the difference between the results given by the two methods lying within the margin of experimental error. Both processes are retarded to the same extent in the presence of guanidine.

Table II. *Comparison of results obtained nephelometrically with those obtained by Bertrand's method.*

Time mins.	Percentage decomposition				Retardation (%)	
	Without guanidine		With guanidine			
	Nephelometer	Bertrand	Nephelometer	Bertrand	Nephelometer	Bertrand
10	6·0	—	3·0	—	50·0	—
20	12·0	—	8·0	—	33·3	—
30	20·0	22·3	16·0	16·1	20·0	27·6
50	27·0	27·5	24·0	24·3	11·1	11·0
70	34·0	34·0	30·0	30·5	11·7	10·2
90	42·0	43·9	38·0	40·1	9·2	8·6

3. *Action of creatine.*

In experiments on the action of amylase in the presence of creatine or creatinine only nephelometric measurements were made, as both of these substances reduce cupric oxide in Bertrand's reagent. The systems consisted of 20–25 cc. of 1 % creatine, 20–25 cc. of 1 % glycogen, 10–20 cc. of Sørensen's buffers (p_H 5·88–7·39), and water to 97 or 99 cc. The systems were warmed to 34°, and 1–3 cc. of a 0·1 % solution of saliva was added, after which glycogen was determined at 10 min. intervals.

Table III. *Effect of creatine.*

No. of exp.	Conc. of glycogen %	Conc. of creatine %	p_H	Minutes 10	20	30	40	50	60	70	
1	0·25	0	5·88	45	68	—	—	—	—	—	% hydrolysis
	,,	0·25	,,	38	60	—	—	—	—	—	,,
	,,	,,	,,	15·5	11·5	—	—	—	—	—	% retardation
2	0·20	0	6·8	30	53	61	—	—	—	—	% hydrolysis
	,,	0·20	,,	28	51	65	—	—	—	—	,,
	,,	,,	,,	6·6	3·7	8·4	—	—	—	—	% retardation
3	0·25	0	7·22	—	14	24	33	—	—	43	% hydrolysis
	,,	0·25	,,	—	14	24	33	—	—	43	,,
	,,	,,	,,	—	0	0	0	—	—	0	% retardation
4	0·2	0	7·39	32	55	67	—	—	—	—	% hydrolysis
	,,	0·25	,,	31	52	65	—	—	—	—	,,
	,,	,,	,,	3·1	5·4	3·0	—	—	—	—	% retardation
5	0·2	0	6·0	20	26	32	40	48	54	—	% hydrolysis
	,,	0·4	,,	14	20	26	32	38	46	—	,,
	,,	,,	,,	30	23·1	18·8	20·0	20·8	18·6	—	% retardation

The results (Table III) indicate that creatine considerably retards reaction in acid media, and to a much less marked degree or not at all in alkaline media. Its inhibitory effect is, however, much smaller than is that of guanidine.

4. *Action of creatinine.*

Systems similar to those described in the preceding experiment were set up, with the difference that creatinine (in final concentrations of 0·4–0·8 %) was taken in place of creatine. The results (Table IV) indicate that the reaction

Table IV. *Effect of creatinine.*

No. of exp.	Conc. of glycogen %	Conc. of creatinine %	p_H	Time in minutes 10	20	30	40	50	60	70	
1	0·2	0	6·27	25	40	50	60	—	—	—	% hydrolysis
	,,	0·4	,,	40	58	74	80	—	—	—	,,
	,,	,,	,,	60·0	45·0	48·0	33·3	—	—	—	% acceleration
2	0·2	0	6·3	25	42	54	—	—	—	—	% hydrolysis
	,,	0·8	,,	60	77	90	—	—	—	—	,,
	,,	,,	,,	140	83	66	—	—	—	—	% acceleration
3	0·2	0	7·01	14	25	31	40	46	50	56	% hydrolysis
	,,	0·4	,,	15	30	42	48	55	62	69	,,
	,,	,,	,,	7·1	20·0	32·6	20·0	24·0	24·0	23·0	% acceleration

proceeds with a considerably greater velocity in the presence of creatinine than in its absence, and that percentage activation increases with concentration of creatinine.

5. *Action of amylase in the presence of both activating and inhibiting substances.*

. The activator taken was glycine, whilst guanidine carbonate was selected as an inhibitory factor. The systems consisted of 20 cc. of 0·5 % starch, 10 cc. of buffer solution (p_H 5·8), 10 cc. of water or 0·5 % guanidine carbonate, 2·5 % glycine in such quantities as to give a range of concentrations of 0 to 1 %, and 2 cc. of 0·1 % amylase, added after the rest of the solution had been warmed up to 27°. Reducing sugars were determined in all systems after keeping for 1 hr. at 27°. The results (Table V) indicate that a retardation of reaction amounting to 29·1 % is brought about by addition of guanidine, whilst glycine accelerates reaction by from 7·6 to 26·8 %, according to its

Table V. *Simultaneous action of guanidine and glycine.*

	Without guanidine		With guanidine	
Conc. of glycine %	Maltose mg.	Acceleration %	Maltose mg.	Retardation %
0	15·8	—	11·2	29·1
0·25	17·0	7·6	12·4	21·5
0·50	18·1	14·5	13·4	15·2
0·75	19·2	21·5	15·2	3·8
1·00	20·0	26·5	16·6	− 5·0

concentration. Retardation due to guanidine diminishes progressively with increase in concentration of glycine, in 1 % solutions of which the reaction is accelerated by 5 %. It appears, therefore, that the processes of retardation due to guanidine, and of acceleration due to glycine proceed independently of each other, and that the resultant effect is the algebraic sum of the effects due to each factor separately. Thus guanidine in the concentration taken retards reaction by 29·1 %, whilst 0·25 % glycine accelerates it by 7·6 %; where the two substances are present together, the resultant retardation amounts to 21·5 %, or 7·6 % less than in the presence of guanidine alone. The same applies to systems containing 0·5 % glycine, whilst with 0·75 and 1 % glycine the summation of effects is not so exact.

Summary.

1. The action of guanidine, creatine, and creatinine in enzymic amylolysis has been studied.

2. Guanidine and creatine retard, and creatinine accelerates, the reaction; the effect obtained is proportional to the concentration of the substance studied.

3. The effects obtained are of the same order in the hydrolysis of starch or glycogen by vegetable or by salivary amylase.

4. When both accelerating and retarding substances, *e.g.* glycine and guanidine, are present together, the resultant velocity of reaction is the mean of the velocities which would be obtained in the presence of each substance separately.

The author has much pleasure in acknowledging his indebtedness to Prof. St J. Przyłęcki, at whose suggestion this research was undertaken, for the interest and assistance so freely given by him, and to the Ministry of Education for providing the means for its prosecution.

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OBITUARY NOTICE.

MURIEL WHELDALE ONSLOW.

1880—1932.

MURIEL WHELDALE, only child of John Wheldale, barrister, was born in Birmingham on March 31, 1880. She was educated at King Edward's High School in the same city, a school which at that period held the foremost position amongst girls' schools for science teaching. She entered Newnham College, Cambridge, in 1900, and subsequently took a first class in both parts of the Natural Sciences Tripos with botany as her principal subject. She was assistant lecturer in botany in her own college from 1906 to 1908. Her career as a research student started with a Bathurst studentship in 1904, followed by a Newnham College fellowship in 1909. It was at this period that the trend of her work was strongly influenced by the late William Bateson and her early research was concerned with the inheritance of flower colour in antirrhinums. From this her ambition was fired to pursue the study of genetics in its biochemical aspect. Those same anthocyanin pigments which she had first followed genetically she now worked at chemically, her studies in this field culminating in the publication in 1916 of her well-known monograph, *The anthocyanin pigments of plants*. This book established her reputation both at home and abroad, and deservedly so, for she was amongst the first to visualise and attempt to obtain a chemical interpretation of genetical data.

Her later work, from 1919 onwards, was concerned with the oxidase systems of the higher plants. In addition, from 1917 onwards, she worked for the Food Investigation Board, and from 1922 at the Cambridge Low Temperature Station, directing the chemical section of the team work there in progress on the changes involved in the ripening of fruit. Her results are embodied in the Annual Reports of the Food Investigation Board and will shortly be published in scientific form.

In 1919 Muriel Wheldale married Huia Onslow, who had recently entered the field of chemical genetics, and until his death in 1922 their work was closely associated. In her memoir of her husband (1924) Mrs Onslow has left the record of a man whose amazing courage and mental vitality enabled him to adopt an active biochemical career in circumstances when most people would have subsided into the life of a complete invalid. His great success in the face of incredible difficulties was in no small degree due to the encouragement and assistance he received from his wife. His triumph will always remain an inspiration to those who witnessed it.

Apart from a few years (1911 to 1914) at the John Innes Horticultural Institute, Merton, the whole of Mrs Onslow's career was spent in Cambridge; her activities were divided between the Botany School and the Biochemical Department, where her teaching and research did much to stimulate and maintain an interest in the botanical aspects of biochemistry. Her class in plant biochemistry formed a much valued unit in the teaching of advanced botany.

In 1926 she was appointed to a University Lectureship in Biochemistry, being among the first women upon whom the University bestowed such a form of recognition.

In 1931 her career as a teacher was marked by the appearance of the first volume of her text-book, *Principles of plant biochemistry*; it is a matter of deep regret that her illness and death occurred before the second volume had left her hands.

Through Mrs Onslow's death plant biochemistry is left to mourn the loss of a single-minded and devoted servant whom it can ill afford to spare.

M. S.

CXI. A NEW ERROR OF TYROSINE METABOLISM: TYROSINOSIS. THE INTERMEDIARY METABOLISM OF TYROSINE AND PHENYLALANINE[1].

By GRACE MEDES.

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(*Received March 11th, 1932.*)

THE object of the present paper is to describe an addition to the few known errors of metabolism. That the condition here described for the first time is not common is shown by the following observation. The only way in which it may be routinely discovered is by following up urines reported as giving a doubtfully positive sugar test. Dr Blatherwick, the head of the laboratory of the Metropolitan Life Insurance Co., who was present when some of these findings were presented in Chicago in 1930, has since then followed up 14,753 urines which contained 0·3 % sugar according to Benedict's picric acid method. The test with Fiske's molybdic acid reagent described below was applied to these urines. No instance of our condition was found. One case of alcaptonuria was present.

I. THE HISTORY OF THE INVESTIGATION PRIOR TO THE PRESENT STUDY.

The subject (Max. M., Univ. Hosp. No. 41,154), afflicted with the metabolic disturbance here investigated, is a Russian Jew, aged 49, who during the first half of the year 1927 had been a patient in the Minneapolis General Hospital and had been diagnosed as a case of myasthenia gravis. The reasons for the diagnosis seem adequate and the patient still presents the ptosis, marked on one eye and somewhat less marked on the other, which appears as a characteristic of myasthenia gravis. For four years he has now been free from any acute symptoms of myasthenia gravis. During these years he has been helping with light work of various kinds and during the last year has been working as a repair tailor. He has however a constant creatinuria.

The patient was admitted to the University Hospital July 18, 1927[2], for

[1] Supported by grants from the Research fund of the Medical School of the University of Minnesota.

[2] Through his response to the interests of the Department of Medicine in making possible the uninterrupted hospitalisation of the patient, the Superintendent of the University Hospital, Mr Paul Fesler, has assured himself of our appreciation and demonstrated his realisation of the possibilities of his office in the promotion of clinical research.

the purpose of another investigation [Berglund, Medes and Lohmann, 1927]. During some phosphate determinations by the method of Fiske and Subbarow [1925] in the course of this study, his urine was found to reduce phosphomolybdic acid. This reducing power was further investigated and it was for a while thought of as a possible characteristic of myasthenia gravis [Medes, Berglund and Lohmann, 1927]. Later examinations of urines from more than half a dozen patients with myasthenia gravis have failed to support this assumption. On the basis of the whole behaviour of the anomaly as described in the following pages, it is now believed to be without connection with myasthenia gravis.

Considering the ability of this patient, under conditions described below, to excrete dihydroxyphenylalanine, it seems of importance to state that the patient shows no abnormal pigmentation; particularly that the parts exposed to light show no demarcation in colour from the rest of the body. On exposure of a skin area of the forearm to ultra-violet radiation no abnormal pigmentation developed. The cornea, lens and vitreous humour showed no abnormal precipitates.

II. Methods.

1. Isolation and purification of compounds.

Throughout the experiments, samples of the various compounds were isolated and identified, the methods being varied from time to time according to the conditions of the experiments. The following description applies to the isolation of tyrosine, *p*-hydroxyphenylpyruvic acid, *l*-*p*-hydroxyphenyllactic acid and *l*-3 : 4-dihydroxyphenylalanine which were all isolated from the urine after ingestion of 50 g. of tyrosine.

The urine was treated with an excess of lead acetate and the p_H adjusted to about 5·0 with dilute acetic acid. The precipitate was filtered off and discarded.

The further procedure followed the lines worked out by Guggenheim [1913] and modified by Raper [1926]. The p_H was adjusted to 7·0 with dilute NH_4OH and the precipitate allowed to settle—a process requiring about 3 hours. The clear supernatant liquid, which contained the tyrosine, was decanted and saved for recovery of the latter. The precipitate was centrifuged, washed twice with water and re-centrifuged after each washing. The washings were combined with the supernatant liquid obtained above. The precipitate was suspended in water and decomposed with an excess of H_2SO_4.

The resulting reddish-brown solution was extracted with ether for 24 hours in a continuous extractor, the ether removing the hydroxy- and keto-acids and leaving the dihydroxyphenylalanine. During the process hydrogen was bubbled in a slow stream through the solution to prevent oxidation of the dihydroxyphenylalanine.

Isolation of p-*hydroxyphenylpyruvic acid and* l-p-*hydroxyphenyllactic acid.* The ether extract obtained above contained the keto- and hydroxy-acids, the former highly soluble in the ether, the latter so slightly soluble that when present in large amounts, it crystallised on the sides of the ether chamber of the extractor and could be recovered merely by pouring off the ether. The ether was evaporated slowly, successive crops of hydroxy-acid being collected from time to time. Dilution of the syrupy residue with water caused the keto-acid containing traces of hydroxy-acid to crystallise immediately.

The crystals were removed by centrifuging and freed from hydroxy-acid by washing several times with cold water. The amount of material in the washings was comparatively small and usually no effort was made to recover it.

The *p*-hydroxyphenylpyruvic acid was purified by recrystallising repeatedly from hot water (charcoal), and finally recrystallising from dilute and from absolute alcohol. It separates according

to conditions in needles or rhombic or hexagonal plates. The hexagonal plates are the most stable as the other forms go over to them on standing, M.P. 219°. (Found C, 60·04; H, 4·57 %. Calc. C, 60·00; H, 4·47 %.)

The *p*-hydroxyphenyllactic acid was purified by several recrystallisations from water, followed by one or more from boiling ether. It separates as long silky needles; M.P. 168°. $[\alpha]_{5461}^{20°} = -20·56°$. (Found C, 59·32; H, 5·49 %. Calc. C, 59·30; H, 5·53 %.)

Both compounds give a strong Millon reaction, and were further identified by comparison with synthetic specimens prepared according to Plöchl [1883] and Neubauer [1909].

Through the courtesy of Dr E. C. Kendall I am indebted to Dr A. E. Osterberg of Dr Kendall's laboratory at the Mayo Clinic, Rochester, for elementary analyses of the various compounds isolated.

There is evidence that *p*-hydroxyphenylpyruvic acid exists in two tautomeric states analogous to those described by Bougault and Hammerlé [1915] for phenylpyruvic acid. Although the free acid is characterised by a high degree of insolubility it requires hours to precipitate out of solution when the sodium salt is acidified in the synthesis mentioned above. On the other hand, when the acid is recrystallised from hot water, precipitation occurs immediately upon the slightest cooling at the surface film, but if the acid is dissolved in alkali carbonate and the solution reacidified, precipitation of the acid then requires several hours.

Isolation of 1-3 : 4-*dihydroxyphenylalanine.* The reddish-brown solution remaining after extraction of the hydroxy- and keto-acids was saturated with SO_2 and concentrated in a vacuum desiccator over H_2SO_4 to a sticky mass containing crystals of uric acid and inorganic salts. This mass was extracted with cold water and filtered, by which process the amino-acid was separated from the insoluble residue. The filtrate was boiled with purified charcoal, cooled quickly, again saturated with SO_2 and re-concentrated. Upon repeating this process several times the dihydroxyphenylalanine finally crystallised out and was purified by repeated recrystallisation from hot water. If at any time the solution was left exposed to the air without SO_2, a rapid oxidation took place with the production of melanin. (Found C, 54·29; H, 5·53 %. Calc. C, 54·79; H, 5·62 %.)

The acid crystallises, as first observed by Guggenheim, in thick prisms or fine needles according to conditions, M.P. 270°. It gives a green colour with very dilute ferric chloride, changing to purple with ammonia. Contrary to the statement of Guggenheim, it does not produce a red colour with Millon's reagent but a faint pinkish coloration characteristic of dihydric phenols. I am indebted to Dr Raper for a sample of dihydroxyphenylalanine from *Vicia faba*; its properties were identical with those of the compound obtained from the urine. For the feeding experiments, about 6 g. were isolated from *Vicia faba* in this laboratory and smaller amounts were obtained by the oxidation of tyrosine by tyrosinase prepared from *Tenebrio molitor* [Gortner 1910, Raper 1926, 1927] and extracted and purified according to Raper.

Recovery of tyrosine. The filtrate from the second lead precipitation of the urine (p_H 7·0) contained tyrosine, which was precipitated as the lead salt by adjusting the p_H to about 9·5 with NH_4OH. The precipitate was removed by centrifuging, washed twice with water and the lead precipitate decomposed with H_2S. The PbS was filtered off with suction, the precipitate being washed several times with dilute HCl to dissolve any tyrosine which might have crystallised out. The solution was then boiled with charcoal, neutralised with Na_2CO_3 and concentrated by boiling under reduced pressure. From the resulting dark reddish-brown syrupy liquid impure tyrosine gradually precipitated. It was purified by the method described by Folin and Ciocalteu [1927].

Extraction of melanin. Melanin was not present in the fresh urine of the subject of the present investigation but since dihydroxyphenylalanine and hydroxyphenylpyruvic acid are both potential precursors of melanin, its formation occurred under any condition permitting oxidation. Hydroxyphenyllactic acid is stable and does not oxidise to melanin. Dihydroxyphenylalanine takes up oxygen with such avidity that even saturating the urine with SO_2 does not prevent oxidation, and melanin formation occurred within a few hours after the urine containing this compound was voided. When hydroxyphenylpyruvic acid was present in urine oxidation could be prevented indefinitely by saturating the urine with hydrogen, but, if it were exposed freely to oxygen, melanin gradually separated out. The process required several weeks when the urine was merely left exposed to air, but when oxygen was bubbled through, precipitation was completed in 2–3 days. Warming or rendering alkaline further accelerated the reaction.

To separate the melanin the urine was evaporated and the melanin extracted by the method of Medes and Berglund [1928]. For its purification advantage was taken of the fact that melanin is insoluble in water at its isoelectric point (about p_H 4·0). The acidified methyl alcohol containing the extracted melanin was filtered and evaporated under reduced pressure to a thick syrup which was poured into 5 litres of water, the p_H adjusted to about 4·0, and the solution kept for several days. The melanin collected on the bottom of the flask and was recovered by decanting and centrifuging. It was washed with water, dissolved in anhydrous methyl alcoholic hydrogen chloride and again poured into water. The process was repeated until the melanin was ash-free. Yield, about 3 g. daily.

Products of intestinal decomposition. Search was made from time to time for the presence of other compounds known to represent products of intestinal decomposition of tyrosine. Hippuric acid crystals were observed occasionally, and varied roughly with the amount of tyrosine ingested. They disappeared entirely when the subject was on a tyrosine-free diet. No *p*-hydroxyphenylacetic acid or *p*-hydroxyphenylpropionic acid was found. There is a possibility that traces of some compounds may have been present in the acid lead fraction.

2. *Quantitative estimation of compounds.*

Copper reduction method. In early experiments, before the number and nature of the compounds had been determined, the reducing property of hydroxyphenylpyruvic acid was the outstanding characteristic by which quantitative estimations could be made, and an estimate of the relative amounts present under different dietary conditions was obtained by its reduction of the Folin-Wu [1919] sugar reagent, using the Folin and Berglund [1922] method for normal sugar and employing glucose as a standard. In later experiments the determinations with this reagent were continued in order to obtain an uninterrupted series of estimations made by one method, thereby providing a basis for interpretation of the early findings.

Both *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine reduce this reagent, the equivalents (in aqueous solution) being:

1 mg. *p*-hydroxyphenylpyruvic acid = 0·45 mg. glucose;
1 mg. *l*-3 : 4-dihydroxyphenylalanine = 1·33 mg. glucose.

Since the latter, however, was absent except under high tyrosine feeding, the values were due to the keto-acid *plus* the normal urinary "sugar". In general, no attempt was made to convert the figures into terms of a keto-acid standard, since the error in this determination from the normal reducing substances of urine and from a slight loss of the keto-acid with Lloyd's reagent made it impossible to employ the method for other than relative values.

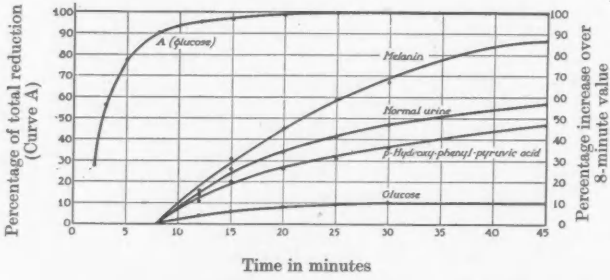


Fig. 1. Reduction of the Folin-Wu blood-sugar reagent. Reduction by glucose (Curve A). Percentage of total reduction at different time intervals. Reduction by melanin, normal urine, *p*-hydroxyphenylpyruvic acid and glucose. Values expressed in percentage increase above their 8-minute values.

The reduction of cupric oxide, while non-specific and hence capable of being used for estimation of other oxidisable substances, is particularly suited for the quantitative determination of glucose since the oxidation of glucose occurs with such rapidity that at 25 minutes it has reached approxi-

mately its maximum value. The percentages of the total reduction after various time intervals as found in one experiment are plotted in Fig. 1 (Curve A). It may be noted that at 8 minutes, the interval of heating as stipulated by Folin and Wu [1919], the curve is straightening. Later Folin and Svedberg [1926] recommended a period of 10 minutes and still later [Folin, 1929], 14 or 15 minutes. At 8 minutes, however, the rate of reduction has become retarded to such an extent that errors of determination due to slight differences in the time interval are only a small percentage of the total value. The same is not true of the various reducing compounds considered in this paper. Their comparative percentages of increase above the 8-minute values are also shown in Fig. 1. It may be seen that the rate of change is higher with *p*-hydroxyphenylpyruvic acid and the reducing substances of normal urine, and still greater with melanin so that a large error may be introduced with slight differences in time. Reduction of the reagent with dihydroxyphenylalanine reaches its maximum in 8 minutes.

Reduction of the Folin and Ciocalteu phenol reagent. This offered another method of estimating the daily output of tyrosine and of its three metabolites, but since the method was not specific it was not used in the metabolism experiments.

Reduction of phosphomolybdic acid. This was used in the form of the Fiske and Subbarow [1925] reagent for the determination of phosphorus. One cc. of urine was measured into a 50 cc. volumetric flask and 5 cc. of the molybdic reagent added. When the unknown was present in pure aqueous solution, 5 cc. of 3·5 % KH_2PO_4 were added to supply the phosphorus. The mixture was then made up to volume and read in the colorimeter against a standard of 5 mg. of *p*-hydroxyphenylpyruvic acid similarly treated with KH_2PO_4 and the molybdic reagent.

Both dihydroxyphenylalanine and *p*-hydroxyphenylpyruvic acid reduce this reagent. Table I shows the rates of colour development and the comparative reducing powers of the two compounds. As may be seen, the keto-acid developed its full colour by the end of 3 hours whereas the amino-

Table I. *Ratio between colour developed by* p-*hydroxyphenylpyruvic acid* (P_2) *with the molybdic acid reagent at various time intervals and the colour of the same compound* (P_1) *after 3 hours. Similar ratio between dihydroxyphenylalanine* (A) *and* p-*hydroxyphenylpyruvic acid* (P_1).

Time min.	$P_2/P_1 \times 100$	Time min.	$P_2/P_1 \times 100$	Time hrs.	$A/P_1 \times 100$
1	39·6	55	87·3	2·5	12·5
3	42·3	70	92·8	5	14·2
5	46·7	85	96·5	23	25·9
10	52·4	105	98·7	24	26·6
15	57·7	120	100·0	27	27·1
20	63·2	180	100·0	30	29·3
25	64·7			51·5	32·5
30	68·8			95·5	35·4
35	69·6			120	38·2
40	73·3			146	35·0
45	79·7			169	30·0

acid required 5 days; its reducing power was then equivalent to 38·2 % of that of the keto-acid. After 2·5 hours, the earliest time at which sufficient colour is developed to obtain a reading with the colorimeter, the colour development was only 12·5 % of the total keto-acid value. Since the amino-acid was present only during the three experiments given here in detail and then in comparatively small amounts, the colour developed at the end of 3 hours was taken to represent the amount of the keto-acid in the urine.

The greatest error in this procedure lay in slight variations in the degree of blueness or greenness of the final colour developed, variations dependent upon the relative amounts of ammonia and other substances in the urine under different dietary conditions. Apart from this difficulty, the determination by this method proved satisfactory since the proportionality of colour developed was good over a wide range.

Another potential source of error lay in possible variations in the relative amounts of the two

tautomeric forms of *p*-hydroxyphenylpyruvic acid and the rate of conversion to the enolic form which is responsible for its reducing properties. According to Bougault and Hammerlé [1915], heating the keto-form of phenylpyruvic acid in the presence of alkali transforms it to the enol derivative. Although heating the urine with alkali destroyed some of the compound, the rate of colour development with the molybdate reagent was unaffected, indicating that the hydroxyphenylpyruvic acid was present in urine almost exclusively in the enol form. These findings explain the failure of our early attempts to identify the reducing compound with a ketone. Repeated efforts failed to obtain a phenylhydrazone, an addition product with $NaHSO_3$ or a semicarbazone.

The Millon reaction. The Millon reaction as developed by Folin and Ciocalteu [1927] proved of special value in both the metabolism studies and the isolation experiments, since molar equivalents of tyrosine, hydroxyphenylpyruvic acid and hydroxyphenyllactic acid all give rise to a similar red colour of about equal intensity. Since the phenols normally present in urine do not produce a red colour with Millon's reagent, this method permitted an estimate of tyrosine *plus* its two metabolites. In some of the experiments the reaction was employed to estimate the amount of tyrosine remaining in the urine after extraction of the ether-soluble compounds from an aliquot part. Thus by combining this method with the molybdate method, which was considered specific for *p*-hydroxyphenylpyruvic acid under the conditions of certain experiments, an estimation of each of the three compounds could be made. The procedure for carrying out the Millon reaction was as follows.

Into a 15 cc. centrifuge-tube were introduced 2·5 cc. urine and 2 cc. 15 % $HgSO_4$ in 6*N* H_2SO_4. This was allowed to stand one hour and centrifuged, after which the supernatant liquid was poured into a 50 cc. centrifuge-tube, the original tube drained carefully and its sides washed with a few drops of distilled water. 2·5 cc. of tyrosine standard containing 1 mg. per cc. in 2*N* H_2SO_4 were prepared in a second 50 cc. centrifuge-tube and 2 cc. of 15 % $HgSO_4$ in 6*N* H_2SO_4 added. To each of these two tubes containing standard and unknown, 10 cc. of 2*N* H_2SO_4 were added and the tubes heated in boiling water for 15 minutes. A second portion of 10 cc. of 2*N* H_2SO_4 was then added to each and the tubes allowed to stand for 30 minutes. If a slight cloudiness appeared in the unknown it was centrifuged again, after which the contents of the tubes were poured into 50 cc. volumetric flasks and 1 cc. of 2 % $NaNO_2$ added to each. They were diluted to volume and read at once in the colorimeter.

This modification of the Folin and Ciocalteu method [1927] was made for the sake of simplicity, and, while some errors were probably introduced, it was found that tyrosine added to normal urine could be determined within 3 %.

III. Metabolic studies[1].

1. Output during fasting.

At three different times the subject fasted for periods of 2 days, each period following a low protein diet. The results, recorded in Table II, show that during starvation, *p*-hydroxyphenylpyruvic acid is excreted to the extent of about 1·6 g. daily (column 4); that this is equivalent to the substances giving the Millon reaction; consequently no tyrosine or *p*-hydroxyphenyllactic acid is excreted, in confirmation of which neither of the two latter nor dihydroxyphenylalanine could be extracted from the urine. It may also be seen that although 3 years elapsed between the earliest and latest determinations there is a marked constancy in the amount of the *p*-hydroxyphenylpyruvic acid excreted. This amount then may be considered as of endogenous origin.

[1] The long metabolism experiments on a variety of diets were made possible only through the generous co-operation of Miss Gertrude Thomas, Head Dietitian of the University Hospital.

Table II. *Urinary output of various metabolites during graded levels of protein intake.*

Date	Total Millon g.	Millon after ether extraction g.	Phosphomol. reduction g.	Cu reduction g.	Creatinine g.	Creatine g.	Total N g.	Amino-N g.	
Fasting.									
Sept. 30, 1927	—	—	—	1·62	0·99	0·53	—	—	—
Oct. 1, 1927	—	—	—	1·56	1·00	0·52	—	—	—
July 8, 1929	1·58	—	1·63	1·63	1·15	0·49	—	—	—
July 9, 1929	1·47	—	1·57	1·36	1·12	0·56	—	—	—
June 3, 1930	1·68	—	1·79	1·57	1·21	0·67	4·07	—	—
June 4, 1930	1·58	—	1·53	1·47	1·25	0·64	3·81	—	—
Average	1·56	—	1·63	1·54	1·12	0·57	3·94	—	—
Mixed diet.									
Exp. 1 (15 days).									
Maximum value	3·04	—	—	3·33	1·35	—	—	—	—
Minimum value	1·89	—	—	1·33	1·02	—	—	—	—
Average value	2·49	—	—	1·97	1·18	—	—	—	—
Exp. 2 (12 days).									
Maximum value	3·28	0·45	—	2·82	1·26	0·87	—	—	—
Minimum value	2·12	0·37	—	1·33	1·05	0·52	—	—	—
Average value	2·74	0·40	—	1·75	1·12	0·64	—	—	—
Exp. 3 (3 days).									
Maximum value	4·68	—	3·28	2·33	—	—	—	0·22	—
Minimum value	3·70	—	2·50	1·76	—	—	—	0·21	—
Average value	4·15	—	2·78	2·02	—	—	—	0·22	—
Exp. 4 (5 days).									
Maximum value	4·08	0·50	3·57	4·01	—	—	—	—	—
Minimum value	2·50	0·37	1·91	2·12	—	—	—	—	—
Average value	3·13	0·44	2·75	2·89	—	—	—	—	—
High protein diet.									
Days of exp.				Cols. 3, 4					
1– 2 (Av.)	2·53	0·45	2·05	2·02	2·50	—	—	—	Hospital diet
3	2·10	0·39	1·75	1·62	2·14	—	—	—	Fasting
4	1·58	trace	1·62	1·56	—	—	—	—	Fasting
5	2·76	0·23	2·47	2·04	2·70	—	—	—	Meat diet
9	3·92	0·71	3·22	3·25	3·93	—	—	—	—
10–12 (Av.)	4·21	0·77	3·20	3·56	3·97—trace hydroxy-acid isolated				

2. Output on a tyrosine-free diet.

On a tyrosine-free diet adequate in calories (cooked starch, gelatin, tea and sugar with small amounts of lemon juice, about 2100 Calories) the subject excreted the same amount of *p*-hydroxyphenylpyruvic acid as during fasting. No tyrosine or other derivative of tyrosine could be isolated. In Table III, days 10 to 13 illustrate such a period; 1·64 g. of the pyruvic compound were found, which comprised all the substance giving the Millon reaction. In Table VI a similar period is recorded. The average output of *p*-hydroxyphenylpyruvic acid was 1·43 g., which corresponded closely with the total tyrosine value. As seen in the table, no hydroxy-acid or dihydroxyphenylalanine or tyrosine could be recovered.

3. Output on a mixed diet.

On a mixed diet the output of *p*-hydroxyphenylpyruvic acid rises from 1·60 g. daily to approximately 2·8 g. daily (Table II, column 4). This amount is no longer equivalent to the total Millon value, the latter showing a greater increase; in Exp. 4, which may be taken as an example, it has risen to 3·13 g. The difference, 0·38 g. represents tyrosine, determined as 0·44 g. on the ether-extracted fractions. There is no longer the same constancy in amount of the keto-acid, the output varying in this series of experiments from 1·91 to 3·57 g. (column 4). The total Millon value shows even wider variations, ranging from 1·89 to 4·68 g. The keto-acid is then both endogenous and exogenous in origin, the tyrosine exogenous.

4. On a high protein diet (Table II).

The output of *p*-hydroxyphenylpyruvic acid rises immediately from 1·62 g., its fasting level, to 2·47 g. and continues to increase to about 3·2 g, which output is maintained over a period of 5 days of the forced diet. The tyrosine, as determined by Millon's reagent on an ether-extracted portion, likewise gradually rose from a trace present on the second fasting day to 0·8 g. During the early days of the experiment, tyrosine and *p*-hydroxyphenylpyruvic acid constituted all of the substance giving Millon's reaction, as may be seen by comparing columns 2 and 4, but as the high protein diet continued a discrepancy between these values appeared, indicating the presence of some other compound included in the total Millon values. Beginning at the 6th day of the high protein diet, traces of *p*-hydroxyphenyllactic acid were isolated. No dihydroxyphenylalanine was present at any time.

5. Ingestion of tyrosine and phenylalanine.

It was shown early in the investigation that tyrosine increased the output of the reducing substance. Table III records a series of these experiments. In each case there was an immediate rise in the copper reduction value over the average value for the preceding days, followed by a falling off on the 1st or 2nd day following. In the first experiment 4·5 g. were given over a period of 4 days. As may be seen, the rise was less, 3·10 g. being the maximum value on the day following ingestion of 2·5 g., after which the output fell to its usual level. Even the ingestion of 0·2 g. produced a prompt elevation over the previous control period and with the low amounts employed here there was no evidence of a cumulative effect.

In order to determine the level of tyrosine intake at which each compound becomes excreted, the subject was placed on a tyrosine-free diet as described above and increasing amounts of tyrosine administered (Table III). During the tyrosine-free period the output of tyrosine *plus* its monohydroxy derivatives fell from an average of 3·21 g. to 1·60 g. (column 2), and of *p*-hydroxyphenylpyruvic acid from 2·75 g. to 1·64 g. (column 4). Tyrosine disappeared from

Table III. *Effect of ingestion of tyrosine.*

Day of experiment	Total Millon g.	Millon after ether extraction g.	Phosphomol. reduction g.	Cu reduction g.	Creatinine g.	*p*-Hydroxyphenyllactic acid (Recovered)	*p*-Hydroxyphenyllactic acid (Computed)	Total N g.	
Exp. 1.									
1– 6 (Av.)	—	—	—	2·97	1·05	—	—	—	Hospital diet
7–15 (Av.)	—	—	—	2·32	1·07	—	—	—	—
16	—	—	—	2·55	1·13	—	—	—	0·2 g. tyrosine
17	—	—	—	2·46	1·05	—	—	—	1·0 g. tyrosine
18	—	—	—	2·96	1·12	—	—	—	0·8 g. tyrosine
19	—	—	—	2·99	1·16	—	—	—	2·5 g. tyrosine
20	—	—	—	3·10	1·17	—	—	—	—
21–23 (Av.)	—	—	—	2·78	1·13	—	—	—	—
24	—	—	—	3·66	1·14	—	—	—	5·0 g. tyrosine
25	—	—	—	2·98	1·05	—	—	—	—
26–32 (Av.)	—	—	—	2·62	1·06	—	—	—	—
Av. control periods	—	—	—	2·52	1·08	—	—	—	—
Exp. 2.									
1– 5 (Av.)	3·21	0·50	2·75	2·89	—	—	—	—	Hospital diet tyrosine-free
10–13 (Av.)	1·60	—	1·64	1·74	—	—	—	6·29	Diet started day 6
14	1·72	—	1·70	1·74	—	—	—	5·94	2 g. tyrosine
15	1·79	Trace*	1·91	2·02	—	—	—	4·28	2 g. tyrosine
16	2·66	—	3·11	2·46	—	—	—	3·62	2 g. tyrosine
17	2·78	Trace*	2·73	2·74	—	—	—	5·12	2 g. tyrosine
14–17 (Av.)	2·24	—	2·36	2·24	—	—	—	4·74	—
18	2·94	—	2·92	2·70	—	—	—	4·90	5 g. tyrosine
19	3·31	†	2·59	2·80	—	—	—	3·19	—
18–19 (Av.)	3·12	—	2·76	2·75	—	—	—	4·05	—
20	5·24	0·74	3·79	3·45	—	—	—	5·40	10 g. tyrosine
21	5·33	0·75	3·83	2·95	—	—	—	3·95	—
22	4·12	0·48	2·67	3·29	—	1·2	—	5·09	—
23	1·98	0·37	1·63	1·46	—	0·6	—	4·92	—
24	2·00	†	1·80	1·73	—	0·3‡	—	5·58	—
25	1·57	†	1·27	1·98	—	Traces	—	4·11	—
26	1·57	—	1·59	2·06	—	—	—	4·22	—
27	1·36	—	—	—	—	—	—	—	—
Exp. 3.									
1– 3 (Av.)	4·21	0·75	3·23	2·61	—	—	—	—	Hospital diet (80 g. protein)
4	7·24	2·14	4·64	3·20	—	0·32	—	—	15 g. tyrosine
5	8·84	2·44	5·20	4·63	—	0·74	—	—	(Urine darkened on standing. 30 mg. dihydroxyphenylalanine isolated impure)
6	6·22	1·32	3·26	2·18	—	0·92	—	—	
7	4·13	0·92	4·09	3·28	—	0·20	—	—	
8	4·28	0·82	3·15	2·50	—	—	—	—	
Exp. 4.									
1– 3 (Av.)	4·15	0·85	2·79	3·02	—	—	0·45	—	Hospital diet (80 g. protein)
4	6·58	1·21	3·95	3·57	—	0·52	1·41	—	10 g. tyrosine
5	7·85	2·16	4·83	3·95	—	0·41	0·86	—	10 g. tyrosine
6	8·83	2·52	5·17	4·82	—	0·73	1·14	—	15 g. tyrosine
7	10·85	3·45	6·43	5·42	—	0·70	0·98	—	15 g. tyrosine (Urine darkened dihydroxyphenyl alanine isolated)
8	8·48	1·00	5·20	5·11	—	1·10	2·28	—	
9	4·58	0·67	2·64	3·09	—	0·80	1·27	—	

* It is not clear whether the colour produced was due to tyrosine or to unextracted *p*-hydroxyphenylpyruvic acid.

† Tyrosine present in small amounts, but the solution cloudy and colorimeter readings could not be obtained. A few crystals were isolated.

‡ Concentration too low to determine accurately.

the urine except for questionable traces. The administration of 2 g. (14th day) produced an immediate rise, which became progressive as similar doses were given on successive days. During this period the *p*-hydroxyphenylpyruvic acid probably constituted all of the substances determined with Millon's reagent as shown by a comparison of columns 2 and 4. The traces of some compounds reacting with Millon's reagent in the ether-extracted fractions were not considered significant, as minute amounts of *p*-hydroxyphenylpyruvic acid could easily have remained unextracted. No crystals of tyrosine or the hydroxy-acid could be isolated.

On an intake of 5 g. of tyrosine there was an immediate increase in the Millon-reacting constituents with a considerable discrepancy on the 2nd day (19th day of exp.) between those and the *p*-hydroxyphenylpyruvic acid. At this time crystals of tyrosine were isolated from the urine.

On the next day 10 g. of tyrosine were administered, with an immediate jump in the *p*-hydroxyphenylpyruvic acid to 3·8 g. and in the total monohydroxy-derivatives to 5·2 g., a level which remained practically constant for 2 days. On each of these 2 days 0·74 g. of tyrosine was excreted. Tyrosine *plus* *p*-hydroxyphenylpyruvic acid amount to about 4·5 g. leaving an unexplained discrepancy between them and the "total Millon." No hydroxy-acid could be isolated. On the 2nd day following the ingestion of 10 g. tyrosine, however, hydroxy-acid began to appear in the urine in amounts large enough to be determined by the optical rotation method. Traces were isolated also on the following day.

The results of this experiment are in agreement with those of the high protein diet, that when the output of *p*-hydroxyphenylpyruvic acid approximates 3 g., tyrosine appears in the urine, and that when the output of the keto-acid is pushed still higher, *p*-hydroxyphenyllactic acid also appears. In the case of the latter, there is a lag in its appearance, in this experiment 48 hours after the ingestion of the tyrosine, a behaviour at variance with that of the other compounds, all of which are excreted at their maximum on the first 2 days.

In Table III Exp. 3 is recorded in which the intake of tyrosine was pushed still higher, 15 g. being fed during a period on high protein diet. The maximum output of "total Millon" reached 8·8 g. on the day following ingestion. The hydroxy-acid, which was possibly present in traces on the 3rd day, was isolated to the amount of 0·32 g. on the day of the tyrosine ingestion, and increased in amount on the 2 following days, recalling a previous experiment in which there was a lag in its maximum excretion. The 3rd day following feeding (day 7 of the experiment) it was noted that the urine was darkening and that the reducing properties also were greater than those of the urine of the previous day. From the urine of this day 30 mg. of dihydroxyphenylalanine were isolated. The dihydroxyphenylalanine, then, is excreted at a still higher level of tyrosine intake than is required for elimination of either tyrosine or *p*-hydroxyphenyllactic acid, and is excreted after a still greater delay.

One further experiment with tyrosine feeding was carried out (Table III, Exp. 4), principally for the purpose of investigating the conditions attending the elimination of dihydroxyphenylalanine as well as for the purpose of securing sufficient crystals for identification. While on a mixed diet somewhat high in proteins, the subject was given 10 g., 10 g., 15 g. and 15 g. of tyrosine on 4 successive days. From the urine of the first day tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid were all isolated. The last, in this instance, was already present possibly in minute amounts owing to the rather high protein diet which had been maintained for 10 days previously. On the 3rd and 4th days after the first tyrosine administration rapid darkening of the urine occurred, and on these two days 100 mg. dihydroxyphenylalanine were isolated. From the combined urines of the entire experiment the following were obtained in highly purified form:

p-hydroxyphenylpyruvic acid	4·217 g.
l-*p*-hydroxyphenyllactic acid	3·162 g.
l-tyrosine	2·086 g.
l-dihydroxyphenylalanine	0·109 g.

Tyrosine and *p*-hydroxyphenyllactic acid can be isolated in pure form almost quantitatively, whereas the purification of the keto-acid is attended with such losses that the final product represents only a small percentage of the amount initially present.

Phenylalanine also increases the output of *p*-hydroxyphenylpyruvic acid. In Table IV are recorded two such experiments. The ingestion of 10 g. of

Table IV. *Effects of phenylalanine ingestion compared with that of tyrosine.*

Date (1928)	Total Millon g.	Millon after ether extraction g.	Phospho-mol. reduction g.	Cu reduction g.	Hydroxy-acid (isolated) g.	
Av. May 26 to 30	—	—	—	2·45	—	Hospital diet
May 31	—	—	—	2·87	—	5 g. phenylalanine
June 1	—	—	—	4·58	—	10 g. phenylalanine
„ 2	—	—	—	3·50	—	—
„ 3	—	—	—	2·67	—	—
„ 4	—	—	—	2·33	—	—
(1930)						
Av. June 2 to 4	3·27	0·47	2·74	2·59	—	—
June 5	3·83	0·51	2·86	2·91	—	10 g. phenylalanine
„ 6	4·52	0·79	3·67	3·23	—	—
„ 7	4·02	0·66	3·04	2·63	Trace	—
„ 8	3·31	0·53	2·58	2·43	0·32	—
Av. June 9 to 12	3·37	0·42	2·59	2·67	—	—
June 13	4·94	0·83	3·85	3·90	—	10 g. tyrosine
„ 14	5·15	0·63	4·41	3·54	—	—
„ 15	3·82	0·31	3·03	2·67	0·41	—
„ 16	3·24	0·41	2·90	2·59	—	—

phenylalanine causes an immediate rise of monohydric phenols as shown by the total Millon, a rise somewhat slower and more prolonged than that pro-

duced by tyrosine under similar conditions as shown later in the table. The same holds true for the excretion of tyrosine (column 3). Tyrosine causes a somewhat greater rise in the *p*-hydroxyphenylpyruvic acid, this increase also occurring on the first 2 days. *p*-Hydroxyphenyllactic acid is excreted only on the 2nd day following tyrosine ingestion, whereas in the phenylalanine experiment it appears only in traces on the 2nd day and is excreted in maximum amount on the following day.

A calculation of the excess of these three substances excreted above the expectancy, as estimated in each control period, shows that following phenylalanine feeding an excess of 4·37 g. was excreted, whereas, after tyrosine ingestion, an excess of 8 g. was excreted, *i.e.*, in the former case 5·63 g. were unaccounted for and in the latter 2 g. It is possible that part of the phenylalanine was excreted unchanged.

Glycine, tryptophan and creatine do not increase the output of the reducing compound in the urine (Table V).

Table V. *Effect of ingestion of various metabolites on the output of* p-*hydroxyphenylpyruvic acid.*

Volume cc.	Creatinine g.	Sugar g.	
2155	1·10	2·64	Hospital diet
2395	1·08	2·37	—
1985	1·05	2·25	—
1940	1·10	2·42	—
2585	1·02	2·64	5 g. glycine
2940	1·00	2·12	—
2240	1·13	2·64	—
1880	1·11	2·58	—
2400	1·10	2·48	—
2150	1·14	2·49	5 g. glycine
2325	1·01	2·34	—
2250	0·97	2·21	—
1760	1·07	2·26	—
2270	1·21	2·35	15 g. tryptophan
1950	1·13	2·61	—
1920	1·26	2·49	—
1970	1·09	2·66	—
1975	1·07	2·60	5 g. creatine
1660	1·25	2·46	5 g. creatine
1820	1·33	2·54	10 g. creatine
2350	1·40	2·38	10 g. creatine
2500	1·19	2·61	—

6. Response to the various tyrosine derivatives.

In the following series of experiments the three metabolites of tyrosine, *p*-hydroxyphenyllactic acid, *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine were fed. The results are given in Table VI.

(*a*) p-*Hydroxyphenyllactic acid.* 2 g. of *p*-hydroxyphenyllactic acid were fed on each of 2 successive days, the subject being on a tyrosine-free diet. None of the compound was recovered the first day, a fact in accord with the good agreement between the "total Millon" and the *p*-hydroxyphenylpyruvic

Table VI. *Effects of ingestion of* p-*hydroxyphenyllactic acid,* p-*hydroxyphenylpyruvic acid and dihydroxyphenylalanine.*

Day of experiment	Total Millon	Phospho-mol. reduc-tion	Cu reduc-tion	Millon after ether extrac-tion	Recovered (impure) Keto	Hy-droxy	Dihy-droxy phenyl-alanine	Tyro-sine	Diet
1	2·15	1·94	1·66	0·85	1·03	—	—	Trace	Tyrosine-free
Maximum (4–8)	1·81	1·49	2·79	0·30	0·73	—	—	—	—
Minimum (4–8)	1·40	1·33	1·15	—	0·56	—	—	—	—
Average (4–8)	1·53	1·43	1·68	—	—	—	—	—	—
9	1·30	1·31	1·64	—	0·67	—	—	—	2 g. *p*-hydroxyphenyllactic acid
10	2·00	1·39	2·06	—	0·62	0·82	—	—	2 g. *p*-hydroxyphenyllactic acid
11	1·71	1·59	2·17	—	0·71	0·64	—	—	—
12	1·17	1·03	2·75	—	0·69	0·22	—	—	—
13	1·37	1·36	1·70	—	0·76	0·10	—	—	—
14	1·19	1·20	1·44	—	0·62	—	—	—	—
15	2·18	2·20	2·14	—	1·09	—	—	—	4 g. *p*-hydroxyphenylpyruvic acid
16	3·85	2·62	2·09	—	1·47	1·04	—	—	4 g. *p*-hydroxyphenylpyruvic acid
17	1·56	1·42	1·33	—	1·21	0·21	—	—	—
18	1·83	1·67	1·72	—	0·96	0·36	—	—	—
19	2·03	1·79	1·94	—	0·97	0·33	—	—	—
20	1·81	1·70	2·34	—	1·20	0·11	—	—	—
21	2·08	1·71	2·34	—	1·15	—	—	—	—
22	1·69	1·67	2·29	—	0·64	—	—	—	—
23	1·00	1·17	1·75	—	0·50	—	—	—	—
24	1·10	1·50	1·56	—	0·52	—	—	—	—
25*	1·20	1·37	2·22	—	0·54	—	46	—	2 g. dihydroxyphenylalanine
26*	1·18	1·39	1·60	—	0·60	—	141	—	—
27*	1·93	1·76	1·79	—	1·20	—	529	20	—
28*	4·09	3·15	3·31	—	1·76	—	101	57	—
29†	2·19	1·92	1·40	—	—	—	—	+	—
30	1·75	2·25	2·55	—	1·46	—	—	43	—
31	2·51	2·19	2·62	—	1·04	—	—	—	—
32	1·57	1·56	2·94	—	0·61	—	—	—	—
33	1·43	1·69	2·45	—	—	—	—	—	—
34	—	1·43	2·00	—	—	—	—	—	—
35	—	1·30	1·44	—	—	—	—	—	—

* Urine blackened on standing. † Collection incomplete. No darkening.

acid as determined by the molybdate reagent. This agreement no longer existed on the following day, when 0·82 g. of the hydroxy-acid was isolated. Its excretion continued over a period of 4 days, during which time 1·78 g. were recovered. No increase in the *p*-hydroxyphenylpyruvic acid occurred except for a doubtful elevation on the 3rd day (column 4). No tyrosine could be detected.

(*b*) p-*Hydroxyphenylpyruvic acid.* 4 g. of this compound were administered on each of 2 successive days. There was an immediate increase in the "total Millon" value, all of which was due to the excreted pyruvic acid. On the second day the output rose to 3·9 g., approximately 1 g. of which consisted of the hydroxy-acid. The latter was recoverable for 5 days, while the increased output of the keto-acid continued over a period of 8 days. No tyrosine could be detected.

(*c*) *Dihydroxyphenylalanine.* On the 25th day of the experiment 2 g. of dihydroxyphenylalanine were administered. There was an immediate excretion of the compound, 46 mg. being eliminated on the 1st day, increasing to 529 mg. on the 3rd, and falling to 101 mg. on the 4th. None could be isolated on the

5th day and there was no tendency for the urine to darken. During the first 2 days the excretion of *p*-hydroxyphenylpyruvic acid was within the limits of the control period, and constituted the whole of the total Millon-reacting substance. On the 3rd day however there was a sudden increase in the keto-acid followed by a still greater increase on the 4th day and a slow decline during the 3 following. On the 3rd–6th days tyrosine was excreted. No hydroxy-acid was detectable at any time. This experiment demonstrates that the oxidation of tyrosine to dihydroxyphenylalanine is a reversible reaction.

7. *Homogentisic acid feeding.*

(*a*) *Comparison of urines containing homogentisic acid and* p-*hydroxyphenylpyruvic acid.* Attempts were made from time to time during the course of these studies to isolate homogentisic acid, but at no time could crystals of the latter be found nor could any reactions typical of homogentisic acid be elicited. Some time later urine from an alcaptonuric was obtained and direct comparisons could be carried out[1]. In Table VII are noted some differences in the reactions of urines containing homogentisic acid and *p*-hydroxyphenylpyruvic acid.

Table VII. *Comparison of reactions of urines containing homogentisic acid and* p-*hydroxyphenylpyruvic acid.*

	Homogentisic acid	*p*-Hydroxyphenylpyruvic acid
Effect of keeping	Darkens	Does not darken
Effect of alkali	Darkens immediately	Darkens slowly
Benedict's qualitative reagent	Reduces immediately to a brownish colour	Reduces slowly to a greenish colour
Phosphomolybdic acid	Reduces at once blue in acid sol. green in alkaline sol.	Reduces slowly dark bluish green
Alkaline silver lactate	Reduces immediately in the cold	Reduces but slightly to a light gray
Ppt. with lead acetate	Ppt. at $p_H = 5{\cdot}5$	Ppt. at $p_H = 6{\cdot}5$

(*b*) *Reactions of homogentisic acid.* Wolkow and Baumann [1891] showed that homogentisic acid reacts with Millon's reagent to give a red colour, since prolonged heating of the acid induces lactone formation between the carboxyl and the *o*-phenolic group. Since none of the reactions used above effects a differentiation between homogentisic acid and the compounds present in the urine of our patient, the simplest tests were employed: the phosphomolybdic acid reduction and the Folin and Wu sugar reagents.

With the phosphomolybdic acid reagent, homogentisic acid develops full

[1] I am indebted to Dr K. K. Sherwood of Redmond, Washington, formerly of this hospital, for first having brought the existence of this case to our attention and to Dr John W. Stuhr of Stillwater, Minnesota, for having brought us in contact with this patient. The patient is a German farmer from near Stillwater, Minnesota.

colour in 4 minutes. The following table gives the percentage of the total reduction effected after various time intervals:

Time in min.	1	1·5	2	2·5	3	3·5	4
Percentage reduction	62	77	85	92	97	99	100

The comparative reducing power of homogentisic acid (4 minutes' standing) and *p*-hydroxyphenylpyruvic acid (3 hours' standing) is:

1 mg. *p*-hydroxyphenylpyruvic acid = 1·33 mg. homogentisic acid.

Homogentisic acid produces its maximum reduction of the Folin and Wu sugar reagent in 8 minutes. The following table gives the percentage of the total reduction effected and the glucose equivalent (mg. glucose = 1 mg. homogentisic acid) after various time intervals. In all cases the glucose standard was heated 8 minutes.

Time in minutes	1	2	3	4	5	6	7	8
Percentage reduction	59	73	81	87	94	98	99	100
Glucose equivalent	0·50	0·62	0·69	0·74	0·80	0·83	0·85	0·86

Table VIII. *Reducing value of urine following ingestion of homogentisic acid.*

	Day of experiment	Volume cc.	Phosphomol. reduction g.	Cu reduction g	
Dec. 28, 1931	1	1080	2·09	1·73	Hospital diet
„ 29 „	2	795	2·15	1·59	—
„ 30 „	3	1505	2·23	1·78	—
„ 31 „	4	840	2·04	1·65	2·5 g. homogentisic acid in 3 doses from 10 to 11 a.m.
Jan. 1, 1932	5	920	2·16	1·70	—
Jan. 13, 1932	1	1055	1·99	1·62	Fasting
„ 14 „	2	840	1·48	1·48	—
„ 15 „	3	890	1·48	1·19	—
„ 16 „	4	390	1·57	1·15	—
„ 17 „	5	750	1·65	1·24	Low protein diet. 5·2 g. homogentisic acid in 4 doses from 10 a.m. to 4 p.m.
„ 18 „	6	700	2·17	1·99	—
„ 19 „	7	1040	1·73	1·27	—
Jan. 21, 1932	1	650	1·50	0·77	Low protein diet
„ 22 „	2	750	1·61	0·92	—
„ 23 „	3	745	1·66	1·11	—
„ 24 „	4	880	1·58	1·01	—
„ 25 „	5	550	1·69	0·99	8 g. homogentisic acid in 8 doses from 9 a.m. to 7 p.m.
„ 26 „	6	1060	1·65	0·98	—
„ 27 „	7	1230	1·60	1·02	—

(c) *Feeding of homogentisic acid.* In the observations recorded in Table VIII, homogentisic acid was administered in increasing doses in three separate experiments. The reduction of the molybdic acid and the sugar reagents was followed, darkening of the urine looked for, and isolation of homogentisic acid attempted. In the first experiment, 2·5 g. homogentisic acid were fed with negative results: no increased reduction of either reagent occurred, no darkening of the urine took place on standing and no homogentisic acid could

be recovered. In the second experiment 5·2 g. of the acid were administered during a period of fasting. On the day following the ingestion there was a slight rise in the reducing power of the urine and, on attempted isolation of lead homogentisate, a few crystals of characteristic shape were observed under the microscope. They were too few for positive identification. A third experiment was made with 8 g. homogentisic acid, an amount which has been found to be slightly more than the maximum completely assimilable by a normal individual. No increase in the reducing power of the urine occurred and no homogentisic acid was recovered. This experiment furnishes convincing evidence that this subject is able to oxidise 2 : 5-dihydroxybenzene derivatives, with the same ease as can normal individuals.

IV. Melanin ingestion by a normal individual.

As has been mentioned before, the isolated keto-acid can be oxidised to melanin. An experiment has been performed to see whether this melanin when ingested is metabolised or is excreted unchanged. Accordingly, 0·5, 3 and 5 g. of melanin were fed to a normal person.

As a preliminary experiment it was determined that the melanin is not digested by trypsin. The amount of reducing substance in the urine was estimated by the Folin-Wu sugar reagent and the subject was put on a diet approximately carbohydrate-free to reduce the normal "sugar" value of the urine to the lowest limit. The tubes were kept in the boiling water-bath for 20 mins. At this interval, the reducing power of the purified melanin compared with that of glucose is: 1 mg. melanin = 0·047 mg. glucose. The colour of the urines was dark and exact matching of standard and unknown was impossible. Table IX gives the result of the experiment.

The glucose values in column 4 of the table show that there is an increased output of reducing substances following the ingestion of 3 and 5 g. of melanin

Table IX. *Estimation and recovery of melanin from the urine of a normal person fed 0·5, 3 and 5 g. melanin.*

Day of experiment	Melanin fed g.	Volume of urine cc.	Glucose g.	Increase in reduction 0·047	Melanin recovered g.
1	—	855	0·43	—	—
2	—	730	0·50	—	—
3	0·5	720	0·44	—	—
Av.	—	—	0·46	—	—
4	3·0	1120	0·53	2·60	2·01
5	—	730	0·51		
Av.	—	—	0·52	—	—
6	—	740	0·38	—	—
7	5·0	965	0·68	4·94	4·23
8	—	1110	0·46		
Av.	—	—	0·57	—	—
9	—	800	0·33	—	—

from *p*-hydroxyphenylpyruvic acid. This increased output is distributed over 2 days following its ingestion and may be determined almost quantitatively by the Folin and Wu sugar method. Deducting the normal output for 2 days from the 2 days' output after feeding and dividing by 0·047 to convert it to its true melanin value, we find an output of 2·60 and 4·94 g. melanin respectively; 2·01 and 4·23 g. were recovered. Therefore melanin formed from *p*-hydroxyphenylpyruvic acid cannot be metabolised, but is excreted unchanged and may be recovered almost quantitatively.

V. Effects of tyrosine feeding to normal individuals.

Against the background of this knowledge of the intermediary metabolism of tyrosine, a renewed inquiry into tyrosine metabolism in normal individuals was made. The result of feeding of massive doses (50 g.) of tyrosine is shown in Table X.

Table X. *Results of feeding 50 g. of tyrosine to a normal individual in ten 5 g. doses administered between 12.30 a.m. Dec. 1 and 9.30 p.m. Dec. 2.*

			Millon		Sugar		Amino-N	
No. of sample	Period (1930)	No. of hrs.	Total mg.	Per hr. mg.	Total mg.	Per hr. mg.	Total mg.	Per hr. mg.
1	10.30 p.m. Nov. 30–6.30 a.m. Dec. 1	8	—	—	105	13	22	3
2	6.30 a.m.–2.30 p.m. Dec. 1	8	168	20	156	20	37	5
3	2.30 p.m.–10.30 p.m. Dec. 1	8	424	53	145	18	34	4
4	10.30 p.m. Dec. 1–6.30 a.m. Dec. 2	8	266	33	73	9	40	5
	Total	24	858	—	—	—	—	—
	Isolated 0·7 g. *p*-hydroxyphenyllactic acid. 2·1 g. hippuric acid.							
5	6.30 a.m.–4.30 p.m. Dec. 2	10	266	27	185	19	37	4
6	4.30 p.m.–10.30 p.m. Dec. 2	6	391	65	122	20	25	4
7	10.30 p.m. Dec. 2–6.30 a.m. Dec. 3	8	340	43	146	18	26	4
	Total	24	997	—	—	—	—	—
	Isolated 1·6 g. *p*-hydroxyphenyllactic acid. 2·65 g. hippuric acid.							
8	6.30 a.m.–2.30 p.m. Dec. 3	8	269	34	120	15	26	3
9	2.30 p.m.–10.30 p.m. Dec. 3	8	173	22	176	22	41	5
10	10.30 p.m. Dec. 3–6.30 a.m. Dec. 4	8	—	—	115	14	26	3
	Isolated 2·5 g. hippuric acid.							
11	6.30 a.m.–2.30 p.m. Dec. 4	8	—	—	—	—	31	4

A question not touched upon heretofore concerns the degree of absorption of tyrosine when ingested. The question has not seemed of striking importance in the previous case where the results indicated that at least with small amounts the absorption was fairly complete. The degree of intestinal decomposition after ingestion of large amounts by normal individuals is indicated by the isolation of 7·25 g. of hippuric acid (equivalent of 7·25 g. tyrosine) from the urine of the normal individual who took 50 g. of tyrosine in 10 doses over a period of 2 days. The feeding of the tyrosine gave no increase in the amino-acid content of the urine as determined by Folin's amino-acid reagent [1922]. The

urine gave a red colour with the Millon reagent and no colour with the molybdic acid reagent, indicating the presence of hydroxy-acid. From the urine 2·3 g. of *l-p*-hydroxyphenyllactic acid were isolated. No tyrosine could be recovered.

The next question was the determination of the minimum amount of tyrosine required for the appearance of the hydroxy-acid. Table XI records

Table XI. *Excretion of* p-*hydroxyphenyllactic acid by a normal person following ingestion of tyrosine.*

Period of collection	No. of hrs.	Millon: Total output mg.	Millon: Output per hr. mg.
2·5 g. Tyrosine at 8, 9, 10, 11 a.m. (Oct. 6)			
Oct. 4, 8 p.m.–Oct. 5, 8 a.m.	12	66·4	5·5
Oct. 5, 8 a.m.–8 p.m.	12	52·2	4·4
Oct. 5, 8 p.m.–Oct. 6, 8 a.m.	12	62·4	5·2
Oct. 6, 8 a.m.–8 p.m.	12	110·0	9·2
Oct. 6, 8 p.m.–Oct. 7, 8 a.m.	12	150·0	12·5
Oct. 7, 8. a.m.–8 p.m.	12	64·8	5·4
2·5 g. Tyrosine at 9.30 a.m. (March 30)			
2·5 g. Tyrosine at 10.30 a.m.			
March 30, 1931. 6.30 a.m.–9.30 a.m.	3	18·0	6·0
9.30 a.m.–1.30 p.m.	4	28·6	7·2
1.30 p.m.–3.30 p.m.	2	15·4	7·7
3.30 p.m.–6.30 p.m.	3	18·8	6·3
6.30 p.m.–10.30 p.m.	4	20·1	5·0
10.30 p.m.–March 31, 6.30 a.m.	8	48·4	6·1

two experiments in which 10 and 5 g. were taken by the same individual as above. In these experiments the procedure of estimation was varied to increase the delicacy of the Millon test. The subjects were placed on a restricted fluid intake to reduce the volume of the urine and the latter was shaken twice with Lloyd's reagent to remove pigment (Lloyd's reagent does not remove *p*-hydroxyphenyllactic acid). A series of standards was set up with tyrosine in concentrations of 0·1, 0·3, 0·5 and 1 mg. per cc., the procedure being as previously described. The estimations could probably have been made more accurately with the phenol reagent but this procedure was employed for the sake of continuity to obtain comparative results. After the 10 g. ingestion there was an increase in the output of the Millon-reacting substances and traces of the hydroxy-acid were isolated. No rise occurred following ingestion of 5 g.

The regularity of this rise after ingestion of 10 g. tyrosine was investigated by the feeding of this dose to normal individuals. The results are presented in Table XII. A rise in the output of the colour-producing substances invariably occurred. The average Millon value preceding the ingestion of tyrosine was 65 mg. per 12 hour period. In some instances there was no immediate increase, in other cases the output rose to 200–350 % of its original value. In most instances, by the third period following ingestion, values had again fallen to normal, but considerable irregularity was displayed throughout. It is difficult to decide whether these variations are caused by differences in rate of absorp-

Table XII. *Millon reaction of urine of normal persons after ingestion of 10 g. of tyrosine, taken in four 2·5 g. doses at intervals of 1 hour. First dose at beginning of period marked (*). Urine collected in 12-hour periods.*

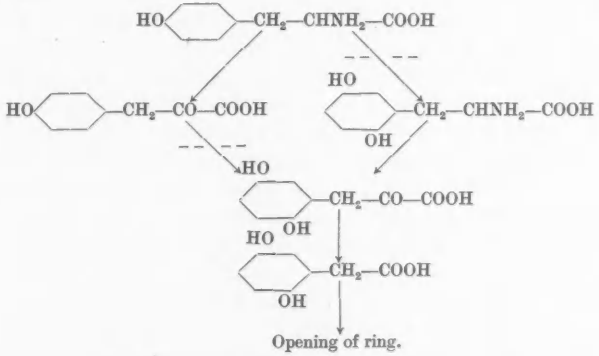
	Millon reaction (tyrosine equivalent of urine)								
	mg.	mg.	mg.	mg. (*)	mg.	mg.	mg.	mg.	mg.
G.M.	67	52	62	110	300	65	—	—	—
R.	75	71	80	150	200	52	—	—	—
R.J.	—	24	75	67	98	—	—	—	—
L.F.	71	63	60	75	182	—	—	—	—
V.	63	50	47	67	143	—	—	—	—
L.	63	22	77	216	342	—	—	—	—
H.	—	—	60	80	81	455	130	—	—
T.B.	—	63	118	135	200	56	—	—	—
H.N.	—	—	47	127	140	91	63	65	50
H.	—	—	26	95	105	77	78	62	57
M.F.	—	—	50	135	141	80	65	—	—

tion or produced by differences in rate of metabolism. That they tend to be characteristic of the individual was shown by repetition of the test in several instances.

With these easily obtainable results in normals it was natural that the ever-recurring search for chemical tests of hepatic function should present itself. Twenty patients with a variety of liver diseases have been tested but no characteristic difference from normal behaviour has been found.

Discussion.

According to Neubauer [1928, p. 862], two paths of oxidation of tyrosine are open to the normal individual, one leading through *p*-hydroxyphenylpyruvic acid and the second through 2 : 5-dihydroxyphenylalanine. Both lead to the production of 2 : 5-dihydroxyphenylpyruvic acid and thence to homogentisic acid.



According to Dakin [1922], the only path open for the oxidation of tyrosine in the normal individual lies through *p*-hydroxyphenylpyruvic acid, in which compound opening of the ring occurs.

(*a*) In the subject of this investigation there is a block in the series of reactions after formation of *p*-hydroxyphenylpyruvic acid. According to the first hypothesis, the error of metabolism involved is an inability to perform 2 : 5-oxidation of the benzene ring; specifically, he cannot oxidise tyrosine to 2 : 5-dihydroxyphenylalanine or the monohydroxyphenylpyruvic acid to the 2 : 5 dihydroxy-derivative. According to Dakin's theory, his anomaly would consist in his inability to open the ring. To test this point he was fed 2·5 g. of homogentisic acid as recorded above. On the two days following its ingestion no darkening of the urine took place on standing or on being rendered alkaline; no significant increase in the reducing power of the urine occurred, and no homogentisic acid could be recovered, demonstrating beyond doubt that our subject is able to open the benzene ring when oxidised in the 2 : 5-position. This experiment leans strongly towards the hypothesis of Neubauer and others, that normal oxidation of tyrosine includes introduction of a second hydroxyl in a position leading to the production of homogentisic acid as a preliminary to disruption of the ring.

The inability of the patient to dispose normally of *p*-hydroxyphenylpyruvic acid is complete, since during fasting his output drops to a constant level of about 1·6 g. which may be regarded as a reasonable figure for the total endogenous tyrosine catabolism. If the block is complete in alcaptonuria, an equivalent amount of homogentisic acid should be excreted under similar conditions. It is with difficulty that from the mass of literature on alcaptonuria, figures for the absolute output of homogentisic acid under fasting conditions can be obtained. Isolation figures of unstable compounds are liable to wide error and in many of the studies of alcaptonuria quantitative estimations have not been corrected to terms of absolute homogentisic acid values. In one of the few such records, Mittelbach [1901] gives the daily output of a fasting male alcaptonuric as 1·7 g. which is the equivalent of 1·8 g. of tyrosine or of the keto-acid, a figure in close agreement with that obtained on this patient.

(*b*) When tyrosine is introduced into the diet at approximately 3 g. daily, tyrosine also appears in the urine. Such an event does not occur in normals. Abderhalden [1912] ingested 150 g. of tyrosine without recovering any, and numerous instances of feeding smaller amounts are on record. The experiments of Kotake *et al.* [1922] indicate that tyrosine in normal rabbits goes over with ease to the keto-acid. The experiments on feeding tyrosine to normals recorded in this paper also show no recovery of tyrosine. With our patient this is not the case, and whenever the intake of tyrosine is slightly elevated, *e.g.* when it is between 2 and 5 g. daily, the capacity of the tissues to convert tyrosine completely to the keto-acid is exceeded and tyrosine itself is excreted. But that this limitation of the conversion capacity is not absolute, but affected by mass action, is shown by the continuous increase in the keto-acid output.

(*c*) At such a time *p*-hydroxyphenyllactic acid also appears in the urine. Dakin [1922, p. 68] is inclined to question Blendermann's [1882] identification

of this acid in the urine of rabbits fed large quantities of tyrosine, but accepts it [p. 90] as the substance found by Schultzen and Riess [1869] in pathological urines. It seems, in fact, to be accepted that this compound occurs rather rarely and usually under pathological conditions.

The experiments with our patient, as well as those with normals recorded above, while confirming the derivation of this compound from *p*-hydroxyphenylpyruvic acid, indicate that the reaction takes place with comparative ease whenever, by mass action effects, the capacity of the tissues to oxidise tyrosine approaches its limit.

The time relations of the appearance of this compound bear out this supposition. In Table IV, following feeding of 10 g. of tyrosine, while the output of the keto-acid and even of tyrosine rose immediately, there was a lag of two days in the hydroxy-acid output, which finally occurred at a time when the peak of excretion of tyrosine and keto-acid had passed. That this lag may be partially due to a certain slowness of the organism in excreting the hydroxy-acid is indicated by the delay in its appearance in the urine after feeding the latter acid itself. These findings bear out Dakin's conclusion that this compound is not an intermediary product in the normal oxidation of tyrosine.

(*d*) The path of oxidation of phenylalanine has been an unsolved question, although evidence has been accumulating that at least one route passes through the tyrosine stage. This evidence consists largely of the findings of Embden and Baldes [1913] that phenylpyruvic acid did not yield acetoacetic acid in the surviving liver and that following perfusion with phenylalanine, traces of tyrosine were isolated. Dakin [1922, p. 86] was unable to confirm this latter finding. In the experiments with our patient, recorded in Table XI, the rise in both the reducing value of the urine and the Millon-reacting substances following administration of phenylalanine was clear cut, although not so prompt or so marked as that following tyrosine ingestion. This evidence strongly supports the theory that phenylalanine is converted at least partly into tyrosine.

(*e*) In our patient, under high tyrosine feeding 3 : 4-dihydroxyphenylalanine appears in the urine. The theoretical importance of this finding is twofold: first, the establishment of the fact that 3 : 4-oxidation of the benzene ring takes place in the body, and second, the isolation of a proved precursor of indole derivatives and of melanin.

From the findings in this investigation, oxidation in the 3 : 4 positions offers some striking contrasts to 2 : 5-oxidation. The latter must take place readily in the normal individual the former with difficulty. There is also a marked difference in the case of the further oxidation of the products, homogentisic acid being broken down completely by the normal individual when ingested in moderate amounts, the 3 : 4-compound appearing in the urine when fed in similar quantities. Guggenheim [1913], for instance, ingested 2·5 g. of the amino-acid and within 5 hours excreted it in sufficient amounts to produce characteristic darkening of the urine and to yield a positive test for catechol

with ferric chloride. Fromherz and Hermanns [1914] believed that they demonstrated that *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine were burned in the body to about the same extent. From our own experiments however, it seems probable that Fromherz and Hermanns were dealing with excreted *p*-hydroxyphenyllactic acid rather than the pyruvic acid. Since the lactic acid represents only a small portion of the pyruvic acid produced by mass action effects, this experiment cannot well be used to compare the relative capacity of the organism to deal with the two substances. The experiments with our subject show difficulty in oxidising 3:4-dihydroxyphenylalanine, which not only fails to be further oxidised to any great extent, but becomes partly reduced to tyrosine. The production of the 2 : 5-compound may be looked upon as a purely catabolic process, while that of 3 : 4-dihydroxyphenylalanine might serve certain specific anabolic purposes.

Tyrosinosis.

The essential feature in our patient, then, consists in a complete stoppage of the oxidation of tyrosine at the stage of *p*-hydroxyphenylpyruvic acid, together with a slowing of every step theretofore. Thus results an excretion not only of *p*-hydroxyphenylpyruvic acid and its reduction product *l*-*p*-hydroxyphenyllactic acid, but also of tyrosine and its oxidation product, *l*-3 : 4-dihydroxyphenylalanine. For this condition, the name tyrosinosis is proposed.

The two errors of tyrosine metabolism now known, alcaptonuria and tyrosinosis, both involve blocks in the oxidation of tyrosine, but at different stages. In tyrosinosis the block occurs following formation of *p*-hydroxyphenylpyruvic acid and consists essentially in the inability of the subject to oxidise the *p*-monohydroxy-compound to the 2 : 5-dihydroxy-derivative. He can, however, open the ring previously oxidised in the 2 : 5 position. In alcaptonuria, the subject is able to bring about 2 : 5 oxidation but cannot further metabolise this compound. He can, on the other hand, oxidise the benzene ring by some other route.

Garrod [1909] suggests that inborn errors are probably caused by "the congenital lack of some particular enzyme, in the absence of which one step is missed, and some normal metabolic change fails to be brought about." It is not easy to explain the slowing of all successive reactions involved in tyrosinosis on this assumption. The formation of *l*-*p*-hydroxyphenyllactic acid is clearly enzymic in character.

It is known that the conversion of tyrosine into dihydroxyphenylalanine is brought about by the action of tyrosinase. Raper states that the enzyme acts only in this first step of the reaction and the further production of melanin takes place spontaneously in the presence of oxygen. In test-tube experiments, the production of the red quinone stage takes place with much greater difficulty in pure solution than when the enzymes from the meal-worm are present and it seems probable that more than one type of enzyme is involved and that the

later stages are also catalysed. According to Bloch [1917] the enzyme present in the melanin-producing cells of the epidermis catalyses only the oxidation of dihydroxyphenylalanine (Dopa). No evidence has been presented as to the method or site of production of the dihydroxyphenylalanine.

Summary.

Under the name of tyrosinosis a new error of tyrosine metabolism in man is described which gives information as to several stages of the intermediary metabolism of tyrosine. Briefly, the condition consists in a slowing of the first steps and a complete stop at the stage of *p*-hydroxyphenylpyruvic acid, but with preserved ability to oxidise homogentisic acid.

The endogenous tyrosine metabolism in this individual results in the daily elimination of about 1·6 g. of *p*-hydroxyphenylpyruvic acid. This compound is excreted entirely in the enol form. As the level of tyrosine metabolism is raised by ingestion of the amino-acid either as protein or in pure form, the *p*-hydroxyphenylpyruvic acid increases and in addition the following substances also appear in the urine: first tyrosine, later *l*-*p*-hydroxyphenyllactic acid and when the tyrosine metabolism is raised still further also *l*-3 : 4-dihydroxyphenylalanine.

Dihydroxyphenylalanine, when fed, is excreted partly unchanged, partly causes elimination of tyrosine and increased elimination of *p*-hydroxyphenylpyruvic acid.

Phenylalanine, when fed, causes elimination of tyrosine, increased elimination of *p*-hydroxyphenylpyruvic acid and excretion of traces of *l*-*p*-hydroxyphenyllactic acid. Whether phenylalanine also appears in the urine was not determined.

p-Hydroxyphenylpyruvic acid, when fed, reappears unchanged and also causes elimination of *l*-*p*-hydroxyphenyllactic acid, but does not give rise to tyrosinuria.

The *l*-*p*-hydroxyphenyllactic acid, when fed, reappears unchanged.

Homogentisic acid, when fed, does not reappear in the urine nor is there any indication of its influencing the elimination of any of the compounds previously discussed.

During no experimental condition did melanin appear preformed in the urine of this individual.

Nitrogen-free melanin prepared by oxidation of the *p*-hydroxyphenylpyruvic acid and ingested by a normal individual was quantitatively recovered from the urine. It was not fed to the individual with tyrosinosis.

This condition is taken to prove the assumed positions of *p*-hydroxyphenylpyruvic acid as one early step in normal tyrosine metabolism, and of *l*-*p*-hydroxyphenyllactic acid as a side-product outside the direct path of reactions. The latter acid is readily formed in normal individuals; its optical activity indicates its enzymic production. The preserved power of the patient

to oxidise homogentisic acid might suggest the 2 : 5 oxidation of tyrosine as a normal intermediary step, impossible for this individual to take—while the next step is impossible for the alcaptonuric.

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CXII. THE FORMATION AND DECOMPOSITION OF URATE GELS.

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Uric acid and urates are usually considered relatively insoluble substances. A few observations have been recorded in the literature of remarkably high concentrations of uric acid in the urine and blood of the fowl [Gibbs, 1929], which were beyond the saturation limits as generally accepted [His and Paul, 1900, 1, 2; Gudzent, 1908]. The explanation seemed to be either the existence of uric acid in an unusual form or in the colloidal condition. That uric acid may be prepared in a high concentration that will gel was first demonstrated by Schade and Boden [1913], using the hydroxide of sodium, potassium or lithium. Whether these preparations are colloidal has been the subject of extended debate [Lichtwitz, 1913; Bechhold and Ziegler, 1914; Gudzent, 1914; Kohler, 1919; Schade, 1922; Keeser and Zocher, 1923; Rona and Meyer, 1923; Freundlich and Loeb, 1927; Ettisch, Loeb and Lange, 1927]. The general consensus of opinion is in favour of a portion of such preparations being in the state of colloidal aggregates. Unfortunately previous workers have not concerned themselves for the most part with the determination of the conditions controlling the formation of such gels. These gels represent supersaturated levels of urate concentration. Before attempting a study of the physiological occurrence of such solutions determination of the major variables controlling supersaturation was first attempted. This has led to a study of uric acid gels and their products of crystallisation.

Experimental.

Uric acid from four different manufacturing houses has been tested in the following experiments. All specimens gave rise to the phenomena studied. Three different sources, mammalian, avian and synthetic, are represented. The results of analyses have shown the moisture content to be negligible (0·1 % or less) and the total nitrogen by the Kjeldahl method to be 33·2 to 33·4 %.

The general method of procedure in the preparation of a gel has been to add

a definite weight of solid uric acid to a definite volume of water. To this mixture were added a few drops of indicator. The material was then heated to the desired temperature and alkali added slowly till the uric acid was dissolved. The resulting solution was cooled and tested for its gelation power as described in particular experiments.

Influence of temperature.

In the preparation of a gel we have frequently met with failure due to a heavy precipitate forming before all the uric acid added originally had dissolved. We have interpreted our failure to maintain a clear solution to the disturbing element of boiling in a supersaturated condition. The example of the preparation of a 1 % solution using phenolphthalein and $N/10$ NaOH may be given. At 100° solution may be almost complete but a heavy white crystalline deposit tends to form rapidly. At about 70° the rate of dissolution is too slow because after several minutes the same effect as at 100° is observed. Between 80° and 90° the uric acid dissolves rapidly and forms a typical clear gel on cooling in an ice-bath. These empirical limits have therefore been adopted in gel formation. The higher the concentration of uric acid, the more unstable is the solution.

The degree of cooling is important to the production of gels. The nature and the concentration of the solute are however modifying factors in this generalisation. Furthermore all gels tend to crystallise but at varying rates.

The following experiment will illustrate these points, using concentrations of uric acid which are minimal and maximal for the particular alkali used. The solutions were obtained by suspending solid uric acid in 100 cc. of water and adding normal alkali to complete solution at 90° and to establish a p_H of 7–8 by phenol red. This solution was rapidly divided into eight parts and suspended in water- or ice-baths at the different temperatures recorded. The various times required for gelation are listed in Table I. The limits of concentration are useful approximations from our own experience of those which will form gels. The p_H level used will be explained in the next section. The time of exposure of the preparation to a high temperature and an alkalinity of p_H 8 should be as short as possible because of the decomposition which is known to occur [Austin, 1907].

Table I. *Influence of temperature on gelation.*

The figures express time in minutes required for the formation of gel or precipitate as indicated.

Temperature °C	NaOH		KOH		LiOH	
	1 %	2 %	1 %	2 %	2 %	5 %
−5	Gel 25	Gel 3	Gel 5	Gel 3	Gel 12	Gel 4
0	Gel 60	Gel 3	Gel 5	Gel 3	Gel 25	Gel 4
10	Ppt. 40	Gel 3	Gel 20	Gel 3	Ppt. 10	Gel 5
20	Ppt. 120	Gel 4	Ppt. 5	Gel 3	Ppt. 30	Gel 10
30	Ppt. 120	Gel 4	Ppt. 5	Gel 4	Ppt. 40	Gel 10
40	Ppt. 120	Gel 5	Ppt. 5	Gel 10	Ppt. 40	Gel 20
50	Ppt. 120	Ppt. 1	Ppt. 5	Ppt. 10	Ppt. 40	Gel 20
60	Ppt. 120	Ppt. 1	Ppt. 5	Ppt. 10	Ppt. 40	Ppt. 20

Several interesting conclusions may be drawn from Table I. The limiting temperature for gel formation is about 40° in the higher concentrations of the three urates. In the lower concentrations it is 0°. If gel formation fails to take place precipitation occurs, denoting a supersaturation at that temperature.

We have also tried ice- and salt-baths at $-10°$, $-15°$, $-20°$, and carbon dioxide snow with ether at $-78°$. Gels will form, at these temperatures but the solvent tends to freeze more rapidly than the gel will form and consequently they are not as suitable as the simple ice-bath.

Influence of surface.

Gelation has not been found to be influenced appreciably by the nature of the containing surface, whether of glass, silica, porcelain, iron or nickel. In presence of small glass beads or of coarse sand the time of gelation of the solution is much prolonged.

Influence of p_H.

Adopting the optimal temperature for dissolution of uric acid and for gelation we may now outline the results of a variation in the hydrogen ion concentration of our preparations. The determinations of p_H have been made colorimetrically by the use of methyl red, bromocresol purple, phenolphthalein, phenolthymolphthalein and tropaeolin O. The values obtained have been frequently checked electrometrically by the hydrogen-calomel half cell method. The limits of hydrogen ion concentration within which gelation will take place have been determined for minimal and maximal concentrations of the urates of sodium, potassium and lithium. They are only sharp to the extent of about 0·5 p_H. Sodium urate will gel between p_H 5 and 8·5, potassium urate between p_H 6 and 11·5 and lithium urate between p_H 6 and 12. We have been unsuccessful in our attempts to cause the gelation of sodium urate in the higher ranges of alkalinity possible with both potassium and lithium urate.

There is to be observed in the region of gel formation a shift to the alkaline side to the extent of 1–2 p_H units as the solution cools and the gel forms. A single experiment over this range will illustrate the change in a 1 % solution of sodium urate prepared at 80° with *N* NaOH and cooled in an ice-bath to 0°.

Initial p_H	5·4	6·2	6·8	7·2	8·7
Final p_H	6·6	7·4	8·4	8·6	9·4

These changes have been noted using bromocresol purple, phenol red and phenolphthalein as indicators. The same shift has been observed in solutions of potassium and of lithium urates under the same conditions and to about the same degree. With sodium and potassium urates the gels form rather quickly, frequently before the solution has cooled to the temperature of the ice-bath. This creates a difficulty in separating the change of indicator due to temperature changes from one which might be caused by a reaction in the urate complex. The gels of lithium urate form more slowly and are more suitable for the

observation of this phenomenon. In a 2 % solution the following changes have been observed, using phenol red as indicator.

Temperature	p_H	Physical state
80°	7·2	Clear solution
0°	7·5	Clear solution
0°	8·4	Firm clear gel
0°	8·4	Crystalline deposit

If the clear gel at p_H 8·4 is melted by raising the temperature a reversal of the shift is evident and the original p_H is re-established at 80°. The main shift takes place during the liquefaction of the gel and with a change in temperature of but a few degrees. Thus in the above experiment 8·4 became 7·5 at 5°. It is to be noted that crystallisation does not alter the p_H.

The temperature coefficients of the indicators must first be considered. Kolthoff [1921] has determined the values for p_H displacement between 18° and 70° to be for phenolphthalein — 0·4, phenol red — 0·3, bromocresol purple 0·0. Our temperature range is between 0° and 80° which should therefore increase these figures slightly. The change is accounted for in part on this basis, as has been observed in the case of lithium urate with phenol red as indicator. That it is not essentially a temperature effect can be seen however with bromocresol purple. We have confirmed electrometrically the observation of Kolthoff that this indicator is not affected by changes in temperature. Hydroxyl ions are liberated as the gel forms. An explanation of this phenomenon is offered in the discussion.

Influence of concentration.

Having established optimal conditions of temperature and p_H, we next worked out on pure solutions the limits of concentration which would permit of gel formation. Below the lower limit the solutions remain clear for several hours on cooling to 0°, then slowly deposit crystals. Above the upper limit the solutions deposit dense precipitates before the uric acid can be dissolved completely and hence represent the limits of supersaturation. These limits are for sodium urate 1–2 %, potassium urate 1–2 %, lithium urate 2–5·6 %. The figures are confirmatory of those found by Schade [1922]. The time required for gelation varies inversely with the concentration and is usually 3 to 60 minutes as is shown in Table I. In some dilute solutions of lithium urate it has been observed to require several hours. The presence of electrolytes and non-electrolytes influences the time and concentration required for gelation, as will be shown in a subsequent paper.

In the process of determining the saturation limits as described above we have noted the amount of *N* NaOH used to obtain complete solution of the solid uric acid at different levels of concentration, at the temperature level of 80–90°, and at the point of neutrality. These figures conform at all levels of concentration to the formation of monosodium urate. The same relationship has been found true for lithium and potassium.

Potentiometric titration of uric acid.

Because of the importance of the p_H in the formation of the supersaturated condition and of gels it was thought that potentiometric titration might yield information of interest in the definition of the limits of free uric acid, mono- and di-basic urates. The assembly used was that of Hildebrand with a Weston model 45 voltmeter of 3 volt scale reading to 5 millivolts with an accuracy of 0·5% by calibration and a Leeds and Northrup pointer galvanometer (No. 2320). We have also used the Cenco-Wendt apparatus with voltmeter scale reading to 10 millivolts. The hydrogen electrode was a modified Hildebrand bell type and it was balanced through an agar bridge by a calomel half-cell containing 1 *N* or saturated KCl. The platinum electrode is poisoned by long contact with the urate solution and it has been our custom to clean the electrode each day by electrolysis in a dilute solution of sulphuric acid and to replatinise frequently.

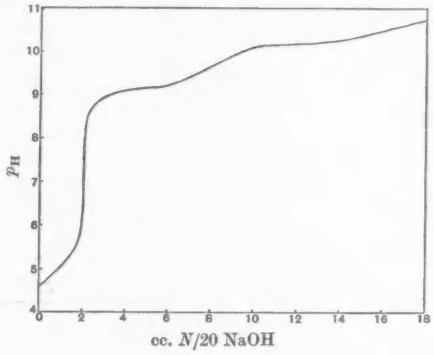

Fig. 1.

Preparations of uric acid for titration have been made in several ways. Initially a saturated solution was obtained by agitating the solid in water for 30 mins. at 20°. Solutions have also been prepared by boiling uric acid with water, cooling and filtering. Titrations have been carried out at 25° and 50°, using 200 cc. of the solution of uric acid and 0·05 *N* NaOH and LiOH as alkalis. Fig. 1 represents a typical titration. The formation of the monosodium urate is complete at about p_H 6 and the disodium salt does not appear under p_H 8·5. Thus in nearly all the experiments discussed in this paper the mono-urate is mainly involved. There is a suggestion of the formation of a trisodium urate in the break of the curve at p_H 10·2.

Because of the low concentration of uric acid in the above experiment an endeavour was made to titrate supersaturated solutions by dissolving solid uric acid in water at 90° by adding 0·5 *N* alkali to the point of complete solution. This preparation was then cooled and the titration continued in the usual

way[1]. LiOH was used because the urate formed does not tend to precipitate out as does the sodium salt. Fig. 2 represents such a titration. The range of the mono-urate is approximately the same, but the formation of the di-urate does not appear. The upper portion of the curve is unusual, as will be seen in Fig. 3. There are three plateaux occurring definitely at p_H 10·6, 10·8 and

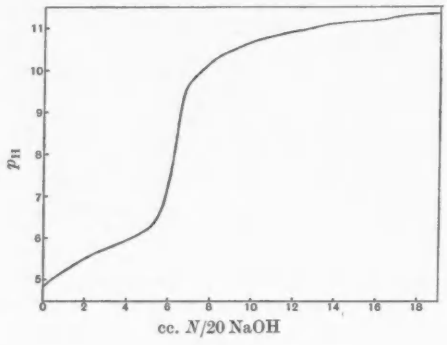

Fig. 2.

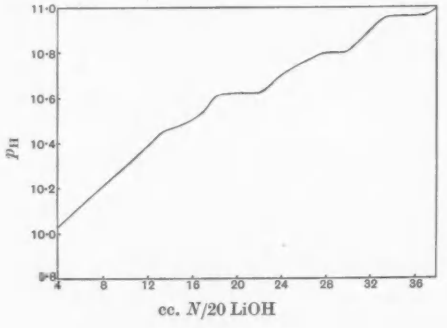

Fig. 3.

10·95. As they have been noticed only in high concentrations which are probably colloidal, the phenomenon is interpreted as an adsorption effect.

Using the expression $p_H = -\log K_a$ at mid-point, the dissociation constants of uric acid have been calculated from the curves. $K_{a_1} = 8 \times 10^{-6}$ and $K_{a_2} = 7 \times 10^{-10}$ as measured in Fig. 1; $K_{a_1} = 3 \times 10^{-6}$ from Fig. 2. K_{a_3} would be in the vicinity of 6×10^{-11}. These figures agree fairly well with those of Kanitz [1921] who found $K_{a_1} = 2 \times 10^{-6}$ and $K_{a_2} = 2{\cdot}6 \times 10^{-9}$ to $8{\cdot}5 \times 10^{-10}$ from data of conductivity.

[1] The authors wish to acknowledge their indebtedness to Mr H. C. Graham for this potentiometric titration.

The influence of organic bases in gel formation.

Schade [1922] has observed that ammonium hydroxide will also form gels with uric acid but that these are the least stable of the group. It was thus anticipated that organic bases would also form supersaturated solutions with uric acid which would gel. An extended list of bases from very weak to strong has been tried. The technique was that developed above.

A known weight of solid uric acid was suspended in 50 cc. of distilled water to which were added a few drops of phenol red. To this suspension at a temperature of 80–90° was added slowly with constant stirring the base as solid, liquid or solution to the complete solution of the uric acid if possible. The rate of addition was such as never to allow the solution to become strongly alkaline and the reaction was maintained between p_H 6 and 8, usually at 7. The resulting solution was filtered if necessary and cooled quickly to 0° in an ice-bath.

In these preparations attention has been paid to the amount of base required to dissolve the uric acid, the character and duration of the gel formed and the composition of the crystalline deposit. The results permit of division of the compounds used into three groups as listed in Table II. The members of the

Table II.

Group 1		Group 2		Group 3	
Substance	K_b	Substance	K_b	Substance	K_b
Ammonia	$1{\cdot}8\times10^{-5}$	Trimethylamine	$7{\cdot}4\times10^{-5}$	Quinoline	$3{\cdot}2\times10^{-10}$
Hydroxylamine	—	Tetramethylammonium hydroxide	>1	Semicarbazide	$2{\cdot}7\times10^{-11}$
Methylamine	$5{\cdot}0\times10^{-4}$	Diethylamine	$1{\cdot}3\times10^{-3}$	Caffeine	$4{\cdot}1\times10^{-14}$
Ethylamine	$5{\cdot}6\times10^{-4}$	Triethylamine	$6{\cdot}4\times10^{-4}$	Creatinine	$7{\cdot}0\times10^{-10}$
n-Propylamine	$4{\cdot}7\times10^{-4}$	Tetraethylammonium hydroxide	>1	Colchicine	$4{\cdot}5\times10^{-13}$
n-Butylamine	$4{\cdot}4\times10^{-4}$	Pyridine	$2{\cdot}3\times10^{-9}$	Benzidene	$1{\cdot}2\times10^{-8}$
Dimethylamine	$7{\cdot}4\times10^{-4}$	Nicotine	$7{\cdot}0\times10^{-7}$	Phenylhydrazine	$1{\cdot}6\times10^{-9}$
Hydrazine	$3{\cdot}0\times10^{-6}$	Ethylenediamine	$8{\cdot}5\times10^{-5}$		
Coniine	$1{\cdot}3\times10^{-3}$	Piperidine	$1{\cdot}6\times10^{-3}$		
p-Phenylenediamine	$1{\cdot}3\times10^{-8}$	Piperazine	$6{\cdot}4\times10^{-5}$		
Guanidine	$8{\cdot}0\times10^{-5}$	Quinine	$2{\cdot}2\times10^{-7}$		
Hexamethylene-tetramine	$2{\cdot}0\times10^{-8}$				

first group form gels with varying degrees of ease. The members of the second group possess the power of dissolving uric acid but, under the conditions outlined, do not form gels. The solutions however will gel in the presence of traces of sodium or potassium chloride. The members of the third group neither form gels with uric acid nor aid in its dissolution perceptibly. For the purpose of comparison of basicity the dissociation constants are also listed in Table II. Ammonia is included for comparative purposes and hydroxylamine was tried because it had never been done before. Obviously basicity is an important factor but it does not account for the different behaviour of groups 1 and 2, nor for the various stabilities of the gels formed. The behaviour of ethylamine preparations is interesting in that an initial cloudiness appears on chilling the solution, which may clear with the formation of a perfectly clear gel which is ultramicroscopically empty, or which may give rise to an opalescent solution, microscopically clear but ultramicroscopically filled with submicrons.

The inability of the powerful quaternary bases to form gels without the presence of electrolyte is noteworthy. Hexamethylenetetramine differs from other bases in several respects. It forms a clear or slightly opalescent, very stable gel which does not crystallise. Gels have been kept for several months at 20° and would seem to keep indefinitely. The temperature of preparation is optimal at 100°. Gels will form almost up to this temperature. They can be melted and solidified repeatedly without precipitation. At 100° there is hydrolytic decomposition of the amine with the liberation of ammonia and formaldehyde. Both compounds have the power to dissolve uric acid at this temperature by chemical action. We have tried to effect gel formation under the adopted conditions with these substances separately and combined without success. Ammonia will form gels only in lower concentrations of urate and the latter precipitates out very readily. Formaldehyde has its characteristic solvent action but the solution will not gel. Moreover the proportion of amine used to the uric acid dissolved is well below that at which either decomposition product will act.

We have found that in the cases of sodium, potassium and lithium hydroxides the ratio is approximately 1 : 1 for the point of complete dissolution of uric acid. Apart from the main reaction involved, this point depends, to a variable extent, upon a number of physical factors, such as rate of addition and volatility of the base, amount of stirring, p_H, *etc.*, and the end-point is not a sharp one. The ratio for the amines forming gels will be found in Table III.

Table III. *Molecular ratio for dissolution.*

Base	Amount Calculated g.	Amount Required g.	Remarks
Methylamine	0·092	0·106	As hydrochloride
Ethylamine	0·134	0·160	As 33 % solution
Propylamine	0·176	0·208	As liquid amine
Butylamine	0·220	0·237	As liquid amine
Guanidine	0·175	0·183	As carbonate
Coniine	0·378	0·387	As hydrochloride
Hydrazine	0·095	0·105	As sulphate
Hexamethylenetetramine	0·417	0·424	As solid base
Hydroxylamine	0·049	0·349	As hydrochloride

The calculated values are based on the molar ratio of 1 : 1. The experimental values are those observed, converted into terms of free base. As might be expected, the required amounts are in all cases slightly higher than the theoretical. The value for *p*-phenylenediamine is not included because of the simultaneous reaction of oxidation. In the case of hexamethylenetetramine the values obtained vary greatly, but the smaller the amount of uric acid used, the nearer to the theoretical does the amount of the amine become in the same volume of solvent. The simultaneous reaction of hydrolysis of the amine is involved here to a variable extent along with two secondary reactions as described above. An exception to the agreement in Table III is hydroxylamine.

The instability of this compound at high temperature is the probable explanation.

Most of the members of the second group require an excess of the base over the 1 : 1 ratio to produce complete solution of the uric acid.

In general it is concluded that uric acid goes into solution at 80–90° in stoichiometrical proportions with the base used as mono-urate.

Composition of crystalline deposits.

The gels formed by using organic bases crystallise at very different rates, from one or two minutes, as in the case of pyridine and quinine, to days or weeks, as with coniine, nicotine, guanidine, piperidine and piperazine. Gels of hexamethylenetetramine have never crystallised spontaneously. The nature of the crystals from the various gels was next investigated. It was hoped to determine thereby whether any chemical reaction was involved in the destruction of the gel. Only the substances of group 1 have been analysed.

The products which resulted from the crystallisation of the gels were filtered off after the process was complete, washed with small quantities of water, alcohol and ether. They were dried first in a desiccator at 20° over calcium chloride and finally in an oven at 105°. The nitrogen values were estimated by the Kjeldahl-Gunning procedure. Complete combustions were not considered necessary because of the limited possibilities of combination and the purity of the products initially.

Table IV. *Elementary composition of crystalline deposits.*

	Nitrogen		Compound ratio	Solution ratio
Substance	Determined %	Calculated %	Base : Acid	Base : Acid
Methylamine	34·6	34·3	1 : 2	1 : 1
Ethylamine	33·3	33·2	1 : 2 (?)	1 : 1
Propylamine	31·9	31·9	1 : 2	1 : 1
Butylamine	30·8	30·6	1 : 2	1 : 1
Guanidine	39·2	39·0	1 : 2	1 : 1
Coniine	27·1	27·3	1 : 2	1 : 1
Hydroxylamine	26·9	27·1	1 : 2	7 : 1
Hexamethylenetetramine	35·0	35·3	1 : 2 (?)	1 : 1
Hydrazine	27·2	—	—	1 : 1

Table IV summarises the results of the nitrogen analyses and compares these figures with the calculated values on the molar ratio nearest to them. In all cases except one the proportion is 1 mol. of base to 2 of uric acid. In the case of ethylamine the figure obtained is consistent with such a proportion but the accuracy of the method is not sufficient to permit of the definite separation from other possible combinations. The hexamethylenetetramine complex is inconstant in its composition. Since the gel did not crystallise it became necessary to obtain the complex by precipitation. This has been accomplished by pouring the warm viscous solution into an excess of ethyl alcohol (95 %) or acetone. Six such preparations yielded nitrogen analyses consistent in themselves but ranging from 30·15 to 37·52 %. Certain preparations have given

figures agreeing with the ratio of 1 : 2 as listed in the table however. Hydrazine furnishes a complete exception in that the product does not yield a figure for nitrogen to be related with any ordinary combination.

Solubility of crystalline deposits.

Because of the very high values obtained for the saturation limit at a temperature of 90°, as shown in Table V for several organic bases, it was considered of interest in certain cases to determine the solubility at 20°. Two

Table V.

	Solubility		
	90°	20°	
		Trituration	Supersaturation
Base	%	%	%
Methylamine	10	0·15	2·38
Ethylamine	8	0·28	1·69
Propylamine	10	1·54	2·88
Butylamine	8	1·47	3·79
Guanidine	1	0·50	0·90
Piperazine	7	0·57	1·41
Piperidine	20	6·65	5·41

methods have been used in obtaining these values, (*a*) the usual trituration for 15–20 minutes and evaporation of an aliquot portion of solution, (*b*) saturation at 40° and cooling at 20° with adequate time allowance for the establishment of equilibrium. The first method yields results considerably lower than the second. The values of the latter tend to be inconstant and to suggest supersaturation with the formation of colloidal aggregates even at low temperatures. This is substantiated by the impossibility of dissolving residues in their original volume of solvent, and the long time required in reaching constant concentrations. By both methods there is always a tendency for the formation of opalescent solutions. Table V illustrates these discrepancies.

The behaviour of the hexamethylenetetramine complex is worthy of note in solubility measurements. It has been found that the degree of solubility depends upon the amount of desiccation to which the urate complex has been subjected. Thus the fresh precipitate is soluble in water to the extent of 0·743 %; if dried for 36 hours in a vacuum, 0·204 %; and if dried completely at 105°, 0·154 %. This phenomenon has also been observed in the case of sodium urate. It is very similar to the behaviour of many proteins.

Discussion.

Apart from the main problem under discussion, a point of interest is the evidence from the experimental details as to the existence of these urates in a colloidal state. In this communication we are not concerned with the attempt to prove or disprove the colloidal nature of our preparations. The instability of high concentrations of urates at 100° is indicative of molecular supersaturation rather than the colloidal condition, particularly since this characteristic

increases with rising concentration to an indefinite limit. The tendency of urates to form supersaturated solutions has been confirmed [His and Paul, 1900, 2] and demonstrated for numerous organic bases. Temperature is an important factor. Indefinite limits of solubility, as noted by earlier workers, [Gudzent, 1908; 1909, 1, 2; His and Paul, 1900, 2], for the alkali urates have also been observed in these cases. The stability of these supersaturated solutions is noteworthy and suggestive of colloidal aggregation. The degree of cooling is important to the formation of the gel. With increasing concentration this factor becomes less important. It is interesting that the temperature of 40° is the upper limit of gel formation. The gel condition inhibits crystallisation for a variable interval of time but appears to be a transitional stage between the condition of supersaturation and the formation of urate crystals.

From the facts which have been presented above we believe that it is possible to suggest an explanation of the phenomenon of gelation in solutions of urates. At elevated temperatures uric acid is dissolved in unimolecular ratio by substances of a basic nature possessing $K_b \leqq 10^{-8}$. These aqueous solutions are capable of a high degree of supersaturation at elevated temperatures and the urates may be said to exist in them in true molecular dispersion so far as is known. These supersaturated solutions become gels on cooling within certain individual limits of concentration, this change being accompanied by a shift of the reaction of the medium to the alkaline side of the original p_H and the gels crystallise. The time required for the crystallisation of the gel is a characteristic of the particular base used. The composition of the crystalline products represents a ratio of two molecules of uric acid to one of base. We may therefore have the following reactions under the conditions specified, taking methylamine as example

$$C_5H_4O_3N_4 + CH_3NH_2 \rightarrow C_5H_4O_3N_4.CH_3NH_2$$
$$2\,C_5H_4O_3N_4.CH_3NH_2 \rightarrow (C_5H_4O_3N_4.CH_3NH_2)C_5H_4O_3N_4 + CH_3NH_2.$$

The acid urate complex is deposited and the amine liberated causes an increase in the basicity of the solution. As has been shown previously, the degree of change is dependent upon the strength of base and the concentration of urate. In all cases the shift is evident during the formation of the gel, and the subsequent crystallisation does not alter the p_H materially. This observation points to the gel being an acid urate rather than the mono-urate and again raises the question of existence of the so-called quadri-urate. Whether or not we are concerned with a true molecular complex, or with the phenomenon of mixed crystals [Ringer and Schmutzer, 1912], or with a solid solution of uric acid in urate [Kohler, 1911], cannot be decided from our experiments. It is significant however that such a variety of urates formed with organic bases should all show the same phenomenon. It is also of importance that this is the type of complex deposited from supersaturated solutions at a p_H of about 7.

In explanation of the fact that two groups of substances exist, the one forming more or less transitory gels with uric acid and the other tending to

crystallise without gel formation, we would recall a paper by Haber [1922]. By a study of solidification from a supersaturated condition using X-ray interference fringes two processes are distinguishable, the formation of unarranged molecular aggregates as amorphous masses and the rearrangement to form crystals depending on the formation of nuclei. These two processes are opposed to one another. Thus if the rate of aggregation is fast, amorphous precipitates result which gradually turn crystalline. If the rate of aggregation is slow as in slight supersaturation, the formation of nuclei may be sufficiently rapid for only crystals to appear. These two processes are evident in our studies, with the modification that the aggregate may appear as a gel in place of an amorphous precipitate. Our first group of urates may be said to aggregate rapidly forming gels, while the second crystallises rapidly and hence does not gel. That the structure of lithium urate gel is non-crystalline has been claimed by Keeser and Zocher [1923] from X-ray analysis. Thus in passing from a supersaturated solution of the mono-urate in molecular dispersion at high temperature a chemical reaction ensues and the particles of acid urate so formed associate in the process of cooling in such a way as to form a gel. How then does this happen?

Haber has further found that the presence of electrical charges on the surface of the original particles modifies the rate of aggregation. An explanation of this behaviour and of gel formation under the conditions of our experiments has been suggested by Usher [1929], who has shown that the presence of an electrolyte such as sodium chloride causes gel formation in gamboge suspensions if in sufficient concentration. The explanation offered is the reduction of the ionic field around the particle under the influence of the electrolyte, so that collisions result in the linear aggregation of particles. These form eventually a mesh-work which is a gel. Usher further states that "a gel is therefore to be regarded as an intermediate stage in the formation of a precipitate...." His photomicrographs are remarkably similar to pictures which we have seen ourselves at times under the microscope with gelatinous precipitates of urate. This would tend to explain urate gels on the micellar theory as a separation of the disperse phase in the form of interlacing fibrils associated with some of the solvent and enclosing the remaining solution enmeshed in the network so produced. Our preparations of gels are optically clear under the ultramicroscope.

The behaviour of urate gels as recorded at present would relate them to the class of colloidal electrolytes such as the soaps as studied by McBain and his co-workers, and such other gel-forming compounds as quinine, eucupine and optochine [Thomas and Sibi, 1929], dibenzoylcystine [Gortner and Hoffman, 1921], benzoyltyrosine, and sorbitol benzoacetal [Thomas and Sibi, 1930].

Summary.

A study has been made of the conditions governing the formation of supersaturated solutions of urates, their gelation and crystallisation. The hydroxides of sodium, potassium, lithium and numerous organic bases have been used to form the urates in unimolecular ratio.

The optimal temperature for supersaturation was found to be 80–90°. The degree of cooling required to produce gelation varied with the concentration of urate between 0° and 40°. The limiting concentrations for gel formation have been determined for the different urates. p_H is an important factor and is limited to 6–8. A shift to the basic side is observable in the formation of gels. The limiting values of p_H relating free acid, mono- and di-urates have been determined by potentiometric titration. The dissociation constants for uric acid have been calculated to be $K_{a_1} = 8 \times 10^{-6}$, $K_{a_2} = 7 \times 10^{-10}$ and a possible $K_{a_3} = 6 \times 10^{-11}$.

The organic bases may be divided into three groups, the first forming gels with uric acid spontaneously, while the second do so only in the presence of certain electrolytes, and the third not at all.

The gels crystallise, with one exception, in the course of a few minutes to several weeks, depending on the nature of the base and the concentration of uric acid used. The crystalline deposits reveal on analysis an acid urate complex consisting of two molecules of acid and one of base.

A hypothesis is advanced in explanation of the phenomenon of gelation and crystallisation in urate solutions.

We welcome this opportunity of acknowledging our indebtedness to the Board of Trustees of the Banting Research Foundation for a personal grant to one of us (F. F. M.).

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CXIII. METABOLISM IN TETANY.

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In a paper published about a year ago Morris, Watson and Morris [1931] made a number of curious statements regarding the work of others and reported some very astonishing results of their own.

Since it seems likely that this paper will only add to the present confusion in the literature, it seems necessary to offer the following remarks.

No attention has been paid to the work of several others [Roos, 1895; Ver Ecke, 1898; Underhill and Saiki, 1908; Greenwald, 1911] on chlorine and creatinine metabolism. Moreover, the references to the other experiments of the present writer are nearly entirely erroneous. Morris, Watson and Morris wrote (page 795): "Greenwald [1922] found a decreased retention of phosphorus during the latent stage following parathyroidectomy and a marked increase during the active period. Recently Greenwald [1929] concluded.... The increase in blood-phosphorus he attributed to a breakdown of lipoids and proteins containing phosphorus."

The first reference is to a paper which does not deal with this subject at all, and the statement does not correctly represent the results obtained. A very marked retention, not a decreased retention was observed, and this retention was frequently continued into the active period.

The second reference is also erroneous. There is no such reference in the paper cited. In another paper, Greenwald and Gross [1923] wrote (page 226): "The concentration of phosphate in the plasma is kept at its normal level or even above that by the phosphate derived from the metabolism of proteins, phosphatides, *etc.*" This, quite obviously, refers to the usual, normal metabolism of these substances and not, as the language employed by Morris, Watson and Morris would seem to indicate, to an abnormal catabolism of lipoids and proteins.

In examining the experimental results reported by Morris, Watson and Morris one notices, immediately, the omission of the *minus* signs before the figures for nitrogen retention on the second, third and sixth lines of Table IV (page 792). These are obvious typographical errors.

The remarkable retentions reported for Cats 3, 4 and 5 deserve some consideration. Cat 3 retained 3·0 g. of nitrogen out of 3·9 g. fed; Cat 4, 2·65 g. out

of 3·53 and Cat 5, 1·80 g. out of 2·61. I do not recall that retentions of such magnitude have ever before been recorded. Certainly they should not be published without drawing specific attention to them.

Apparently, the composition of the food fed to these cats was extremely variable, for, on comparing Tables I, IV and V, one notes an utter lack of relation between the changes in the intakes of nitrogen, chlorine, phosphorus and calcium. An extended analysis is neither necessary nor advisable, but some attention might be paid to the results reported for Cat 3.

	Nitrogen	Phosphorus	Calcium	Chlorine*
Normal	3·90 g.	0·293 g.	0·203 g.	0·707 g.
Active	0·71	0·110	0·057	0·676

* The figures have been rounded off.

The statement is made (page 787) that "the cats received oatmeal porridge and milk." The first explanation for the discrepancies in the reported intakes of the various elements would be that the oatmeal and milk were not ingested in the same proportions on the different days. However, it also appears that the composition of the milk and the oatmeal varied considerably.

Dr S. Morris has kindly sent me the results of some analyses of milk performed "recently." (Date of letter, Dec. 4, 1931.)

Milk	Nitrogen	Phosphorus	Calcium	Chlorine
I	0·5451	0·3109	0·1120	0·1406
II	0·4914	0·3206	0·1175	0·1420
III	0·4298	0·3104	0·1151	0·1349
IV	0·5550	0·3105	0·1253	0·1484

Assuming that these figures represent the nature of the variations in the composition of the milk that occurred during the experiment on Cat 3, certain calculations are of interest. Thus, during the "normal" period, the 0·203 g. of calcium ingested, even if it came entirely from milk, could have been accompanied by no more than 0·99 g. of milk-nitrogen. This amount of milk must have brought with it at least 0·238 g. chlorine. This means that a minimum of $3·90 - 0·99 = 2·91$ g. of oatmeal nitrogen was accompanied by a maximum of $0·707 - 0·238 = 0·469$ g. of chlorine: a ratio of N: Cl that was at least 6·2.

During the "active" period, however, the 0·057 g. of calcium, if it were derived entirely from the milk, would have been accompanied by at least 0·213 g. of nitrogen and by no more than 0·072 g. of chlorine. This means that a maximum of $0·71 - 0·21 = 0·50$ g. of oatmeal-nitrogen was accompanied by a minimum of $0·676 - 0·072 = 0·604$ g. of chlorine, giving a ratio of N: Cl that was no more than 0·83. Even if it be assumed that no milk was consumed the ratio of N: Cl was still only 1·05. It is perfectly obvious that the porridge fed on this "active" day was much more heavily salted than that fed on the "normal" day.

The values for the chloride content of the milk employed in these calculations are those sent me by Dr S. Morris. They are considerably higher than

those usually accepted. However, the use of the more generally accepted values, 0·07 to 0·10 % chlorine, only makes the discrepancies more marked.

Examination of the data for calcium and phosphorus intake discloses still more remarkable discrepancies. The ratios of phosphorus to calcium intake reported by Morris, Watson and Morris were in the order given in their Table V: 1·24, 1·46, 2·38, 0·97, 1·38, 1·18, 1·44, 1·20, 1·93, 1·44, 1·41, 1·51, 1·35, 1·22. In every case these ratios are lower than the ratios of phosphorus to calcium of milk according to the analyses sent me by Dr Morris, and, in every case but two, they are lower than the more usual figure of 1·7. Since any intake of oatmeal could only have raised this ratio to a value greater than that obtaining in milk, it is obvious that the analyses or the calculations employed in the paper under consideration were extremely inaccurate.

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CXIV. AFTERNOON GLYCOSURIA[1].

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(*Received April 16th, 1932.*)

In a previous paper [Harding and Selby, 1931] a phenomenon termed "afternoon glycosuria" was described. This meant that individuals who showed no, or little, fermentable urinary sugar after ingestion of 50 g. of glucose under the usual fasting morning conditions, showed some, or more, after a similar test at 4 p.m. "Afternoon glycosuria" was connected with the height of the blood-sugar curve, for, though at both times the blood returned to its original level in 2 hours, the peak of the curve in the afternoon was usually higher than in the morning.

The amounts of urinary fermentable sugar (and under the conditions this was presumed to be glucose) were small, being often only a few mg. in 2 hours. The morning blood-sugar curves were mainly of the "ideal" type because the subjects were selected as examples of those who had never shown urinary sugar under ordinary clinical tests.

Will "afternoon glycosuria" be observed in those subjects who, though non-diabetic, show clinical urinary sugar, either because they are types of renal glycosuria or cyclic hyperglycaemia? We have subsequently carried out morning and afternoon glucose tolerance tests on subjects who have shown a positive Benedict test for urinary sugar, when examined at the University Health Examination Clinic, and who present no other sign of disability.

Analytical methods.

Blood-sugar. 0·3 cc. cutaneous blood was precipitated in a centrifuge-tube by the method of Herbert and Bourne [1930]. The clear supernatant liquid after centrifuging yields true sugar with about 3 mg. per 100 cc. of non-fermentable reducing material. At the beginning we were troubled by laking and non-precipitation of plasma-protein. This, we found, was due to insufficient stirring of the blood and reagents.

Urinary sugar. Fermentable sugar was determined by the method previously described [Harding and Selby, 1931].

Modified Shaffer-Hartmann solution for sugar determinations. Part of the results was obtained by the use of the modified Shaffer-Hartmann reagent previously described, part by a further modification.

[1] Assisted by a grant from the Banting Research Foundation.

The new reagent is very similar to that described by Spannuth and Power [1931]. A short description has been given by Harding and Downs [1932]. The new solution has the advantage of being more sensitive to glucose and less sensitive to the non-fermentable reducing substances present both in blood and urine.

"Afternoon glycosuria."

The fermentable urinary sugar is shown in Tables I and II. The frequent occurrence of small amounts of fermentable sugar in the fasting urine is to be contrasted with its non-occurrence in the subjects of the previous paper. This

Table I. *Excretion of "fermentable sugar" per 2 hours in subjects showing "afternoon glycosuria."*

Subject	Fermentable sugar, mg. per 2 hours. 7–9 a.m.	*9–11 a.m.	2–4 p.m.	*4–6 p.m.
22 (Nov.)	8	1479	321	3788
22 (Feb.)	20	792	241	2012
13	7	12	393	1213
27	5	0	31	612
31	23	113	105	562
11	0	21	22	309
29	3	18	5	124
6	22	13	10	55
10	2	10	10	53
33	7	3	3	36
26	5	5	11	32
24	0	7	14	21
19	0	6	3	15
20	0	2	0	10
28	0	0	5	5

* 50 g. glucose at 9 a.m.; at 4 p.m.

Table II. *Excretion of "fermentable sugar" per 2 hours in subjects failing to show "afternoon glycosuria."*

Subject	Fermentable sugar, mg. per 2 hours. 7–9 a.m.	*9–11 a.m.	2–4 p.m.	*4–6 p.m.
9	0	17	9	14
16	0	0	9	0
18	0	5	15	4
21	7	—	19	36
23	19	28	16	10
25	5	6	4	7
30	12	0	1	0
1	—	0	13	14
2	0	0	10	5
4	8	—	8	15
12	0	8	4	6
17	0	7	5	0
32	5	6	4	0

* 50 g. glucose at 9 a.m.; at 4 p.m.

probably represents in part a real difference corresponding to the difference in their behaviour to glucose and to the finding of excess urinary reducing substance in the routine examination. Part of it, however, may be due to

improper emptying of the bladder and collection of specimens. We have discussed this previously: these subjects were unused to strict metabolism observations; they corresponded much more closely than our previous group to the ordinary patient in private or hospital practice.

Because of these irregularly occurring amounts of fermentable sugar, we have scrutinised more carefully than formerly the figures on which we have based the conclusion that "afternoon glycosuria" is of common occurrence. Table I shows "afternoon glycosuria" in 14 subjects, representing, in this series, an incidence of 50 %. The phenomenon can evidently be repeated in the same individual, for subject 22 showed it on two occasions, although our previous experience had made us distrustful. Three months, however, had elapsed between the two sets of tests on subject 22. If the corresponding blood-sugar curves of this subject and some of the others be studied, it will be seen that renal glycosuriacs exhibit the phenomenon. Table II contains the results which cannot be interpreted as representing "afternoon glycosuria."

We have found, since writing the previous paper, that "afternoon glycosuria" is known to many of the medical officers of Life Assurance Companies. It is their practice to ask for a urine specimen after the evening meal in doubtful cases.

Morning and afternoon glucose tolerance curves.

In Figs. 1 and 2 are shown the blood-sugar curves of the subjects whose urinary sugar is represented in the tables. All subjects showing "afternoon glycosuria" also show a higher peak blood-sugar curve in the afternoon. In addition there are some who fail to show "afternoon glycosuria," yet who show high peak blood-sugar curves. This is understandable in some of the subjects who show no fermentable urinary sugar either morning or afternoon. In others, although there is fermentable sugar present in the 4–6 p.m. urine, yet it is not certain if this has arisen from the glucose ingested at 4 p.m., or is a continued excretion from the noon meal. Actually 26 out of the 28 experiments show a higher peak blood-sugar curve in the afternoon compared with the morning. In some the difference between the morning and afternoon blood-sugar peaks is very small. In 21, however, the difference is 10 mg. or over.

"Lag" type glucose tolerance curves.

In the previous paper attention was drawn to several blood-sugar curves fulfilling MacLean's [1922] definition of "lag" type. These showed small amounts of fermentable urinary sugar, but not sufficient to be detected by any of the usual clinical tests. It was suggested that the difference between our results and the clinical results was due to the latter being obtained on individuals possessing thresholds for sugar lower than normal.

Our contention is illustrated by subject 22. In November his morning tolerance curve reaches the peak of 187 mg. true glucose, or 212 mg. sugar

as determined by the older methods, and the urine shows clinical sugar. He would thus be classified as a "lag" type, since there is no other disability and he is a healthy vigorous young man. In February a repetition of the

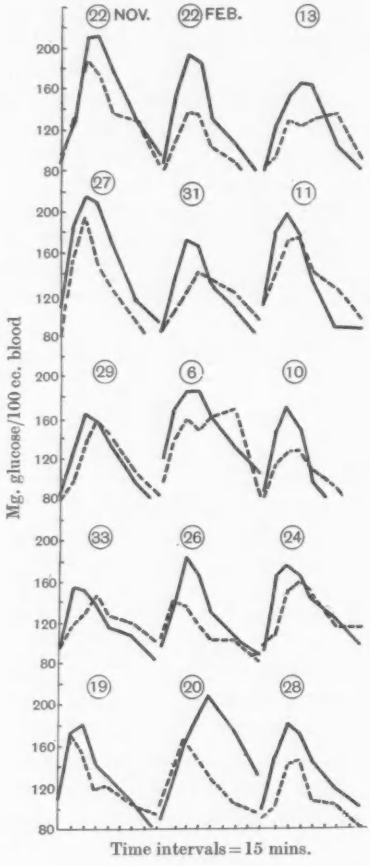

Fig. 1.

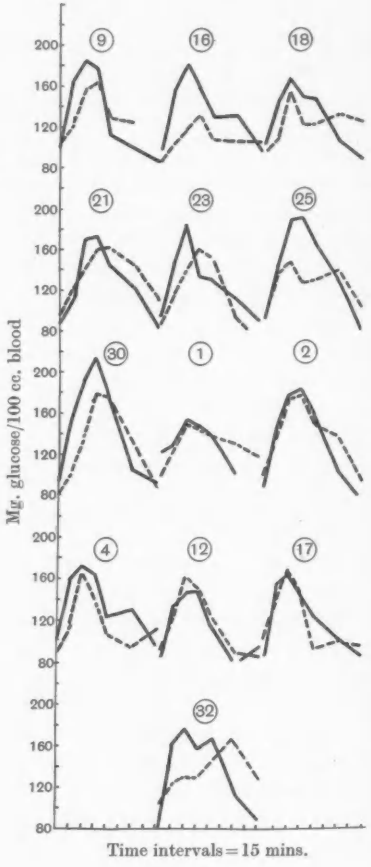

Fig. 2.

Fig. 1. Showing morning and afternoon tolerance curves after 50 g. glucose in subjects showing "afternoon glycosuria."

Fig. 2. Showing morning and afternoon tolerance curves after 50 g. glucose in subjects failing to show "afternoon glycosuria."

Morning curves - - - - - Afternoon curves ———

morning curve gives a result of the "ideal" type, and the urine again shows clinical sugar. He now appears as a renal glycosuriac, of the type recognised by Graham [1923]. We have determined the 2-hourly excretion of urinary

fermentable sugar in this subject for 3 days continuously. At no time is he actually free from urinary sugar, though the amounts before breakfast are too small to be detected by any usual clinical test. His fasting blood-sugar is within normal limits, and he thus appears to possess a threshold slightly below or at this level. On a single glucose tolerance test the accident of a determination in November or in February would classify him either as a "lag" type normal or a renal glycosuriac. Contrast these results with the morning curve of subject 27 who shows a peak of 219 (old standards) and shows no urinary fermentable sugar. Here the sugar threshold is evidently high. Subject 7 (Fig. 3) shows a peak of 237 (old standards) on the morning curve and gives

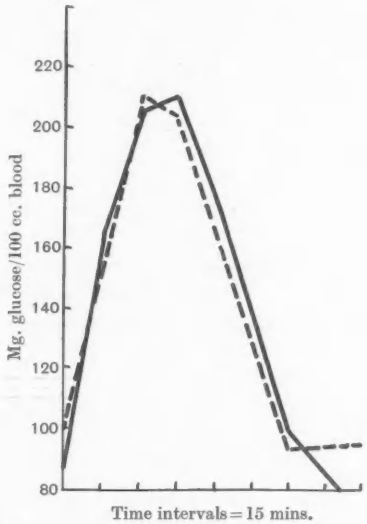

Fig. 3. Showing morning and afternoon tolerance curves after 50 g. glucose in subject 7.

Morning curve - - - - -
Morning glycosuria = 34 mg.

Afternoon curve ———
Afternoon glycosuria = 384 mg.

a urinary fermentable sugar excretion of 34 mg., an amount insufficient to give an ordinary clinical test. It is thus evident that to obtain clinical sugar in the urine of non-diabetics the sugar threshold in the blood must probably be lower than normal to allow of the escape of sufficient quantity. Further point is lent to our contention when we notice that all the subjects quoted by MacLean as of the "lag" type possessed small amounts of clinically demonstrable sugar the hour previous to the glucose tolerance test.

An unusual result.

In the previous paper and in this it has been assumed that "afternoon glycosuria" is related to the difference between the peaks of the morning and afternoon blood-sugar curves. The renal threshold has been assumed to remain constant for the individual. There has been a considerable discussion on this point. Faber [1927] upholds strongly the view of the constancy of the threshold. Many others, as summarised by Peters and Van Slyke [1930], have concluded that at least two thresholds for sugar exist, the first during the ascent of a blood-sugar curve, and a second during the descent.

The data on subject 7 point to the possible variation of the threshold for glucose. Fig. 3 shows the morning and afternoon blood-sugar curves and the urinary excretion. This subject apparently excretes small amounts of fermentable sugar continuously. The amount definitely increases after the morning test. The 2–4 p.m. urine again shows a little fermentable material. The afternoon blood-sugar curve is so similar to the morning curve that it is difficult to imagine two curves on the same individual showing greater agreement. Yet the afternoon urinary sugar excretion is over 10 times the morning excretion. In our present state of knowledge we can only state that the threshold appears to have altered. Hamburger [1919] has demonstrated variation to sugar permeability in the kidney of the frog under experimental changes in the ionic concentration of the perfusate, but so far such results have not been observed in the human organ. The possibility of a diurnal change of this character complicates enormously the explanation of the physiological glycosurias.

SUMMARY.

1. "Afternoon glycosuria" has been found in 14 out of 28 subjects, who showed "sugar" on routine urine examination. Renal glycosuriacs can show "afternoon glycosuria."

2. Over 60 % of the total subjects show a blood-sugar peak after 50 g. glucose higher at 4 p.m. than at 9 a.m.

3. One subject showing almost identical a.m. and p.m. glucose tolerance curves, gave marked "afternoon glycosuria." It is assumed that alterations in the renal threshold for glucose occur during the day in some subjects.

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CXV. THE RELATIVE PROPORTIONS OF FERMENTABLE AND NON-FERMENTABLE REDUCING SUBSTANCES OF HYPERGLYCAEMIC BLOODS OF DIABETICS WITHOUT GLYCOSURIA.

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Claude Bernard first showed that sugar is a normal constituent of blood and recognised that glycosuria is, in some manner, dependent upon hyperglycaemia. Since then a vast literature has accumulated on the subject and though the consensus of opinion favours the view that there is a renal threshold for sugar this view is not, as yet, unanimous. Thus, according to Benedict *et al.* [1918], urine normally contains glucose which is excreted continuously, the process being termed glycuresis. On the other hand, Folin and Berglund [1922] claimed that in the absence of emotional complications ingestion of as much as 200 g. of glucose did not cause the appearance of sugar in the urine of normal persons and concluded that glycuresis represents absorption and excretion of foreign unusable carbohydrate materials present in the ingested food. More recently, however, Hassan [1928] was able to prepare glucosazone from many samples of normal urine and concluded that failure to detect glucose in the past was due to technical difficulties. The writer repeated Hassan's work and obtained somewhat similar results (unpublished data). In quite an exhaustive study, on the other hand, Harding and Selby [1931] concluded that within the limits of analytical methods, fermentable sugar is absent from normal fasting urine. Analysis of all of the data, however, shows that Hassan's results, those of Harding and Selby and our own are not incompatible with each other. Thus, Hassan's data, as well as our own, clearly indicate that though normal urine may have contained glucose, detection of this sugar depended upon the time elapsing between collection of the urine and the last meal; as the fasting state was approached, the urine contained less and less sugar. This agrees with the findings of Harding and Selby who observed that, though there was no glycosuria in the fasting state, 50 % of medical students showed small amounts of fermentable sugar in the post-prandial afternoon urine.

The finding of sugar in normal urine appears at first to be incompatible with the renal threshold concept. Experiences with blood-sugar-time curves, however, suggest otherwise. Frank [1913] first made the observation that

sugar may appear in the urine at a much lower level of blood-sugar concentration when the sugar content of the blood is decreasing than when it is increasing. It was because of the quite frequent occurrence of this phenomenon that attention was called to the unreliability of blood-sugar-time curves when used alone for the diagnosis of renal glycosuria [Rabinowitch, 1930]. It is suggested that this phenomenon explains the finding of sugar in normal urine in the absence of hyperglycaemia and that the necessary condition is a temporary increase of blood-sugar above the renal threshold level. Such increase is probably a common phenomenon following an ordinary meal, but would be detected only with repeated blood-sugar examinations at very short intervals of time. Once the blood-sugar has reached a level above the renal threshold, sugar excretion begins and continues for a short period of time, in spite of the subsequent decrease of blood-sugar. Ingestion of a large amount of sugar is not necessary. Experiences with blood-sugar-time curves indicate that the height to which the blood-sugar rises following glucose ingestion is not strictly proportional to the amount of sugar given. Small amounts may suffice to raise the blood-sugar; with as little as 5 g. an appreciable increase may be noted, and with doses exceeding 25 g. the peak of the curve is not materially affected. Increasing the amount of sugar affects chiefly the rate of decay of the curve; it prolongs the time necessary for the blood-sugar to return to the normal level.

The above are a few examples of the many possible physiological variables which are met with in renal threshold studies, and it is perhaps these difficulties which have made it doubtful whether the renal threshold concept is at all applicable to any blood constituent. Himsworth [1931] has recently reviewed the different methods now in use for determination of the renal threshold for glucose and dealt with some of these difficulties; a new method was suggested.

For practical purposes it is expedient to assume that there is a renal threshold for glucose; that glycosuria, with rare exceptions, is due to hyperglycaemia. (Glycosuria here implies sugar in sufficient concentrations to be detected by the ordinary copper reduction methods, Fehling's, Benedict's, *etc.*). Otherwise one could not differentiate between such conditions as renal glycosuria and diabetes mellitus. The latter, as is well known, usually proves fatal in the absence of treatment, whereas the former requires no dietetic restrictions and is perfectly compatible with good health and long life.

To support the renal threshold concept, there is the experience not infrequently met with in diabetes, namely, marked hyperglycaemia without glycosuria detectable by the ordinary reduction methods; the blood-sugar in such cases may amount to 0·2–0·4 % or more. This phenomenon was first observed by von Noorden [1917] in a case of pneumonia where the blood-sugar was 0·280 %. Blood-sugar concentrations over 0·25 % without glycosuria have been recorded by Graham [1917], Sherrill and John [1922], John [1927], Allen [personal communication] and Joslin [1928]. Many more examples have

been met with more recently. What is believed to be a classical case is that reported by Rabinowitch [1929], a blood-sugar of 0·825 % being found in the absence of glycosuria. This was a case of diabetic coma with marked kidney damage; the blood-urea-nitrogen was 81 mg./100 cc., the creatinine was 5·66 mg./100 cc., and the diazo-reaction for uraemia was positive. A blood-sugar of 0·769 %, without glycosuria, was found in the same patient 3 hours previously. Stone [1926] regards this raised renal threshold as an indication of unfavourable prognosis; autopsy findings indicated hyalisation of the islet tissue.

The conditions in which a raised renal threshold is usually found in diabetes are (*a*) gross dietary indiscretions, (*b*) diabetes of long duration, (*c*) chronic nephritis, (*d*) arteriosclerosis and (*e*) infection. Insulin was found to be a factor [Rabinowitch, 1924]. Major and Davis [1925] later reported seven cases of raised renal threshold in patients without arteriosclerosis or renal disease, but who were taking insulin. The rapidity with which the condition may appear following institution of insulin treatment was previously dealt with [Rabinowitch, 1926].

In a previous communication [Rabinowitch, 1928] the writer dealt with the non-fermentable reducing substances of blood in diabetes and it was found that, ordinarily, they are of no significance; the average values found in 100 normal adults, 10 normal children and 100 diabetics were approximately the same, namely, 0·025, 0·023 and 0·027 % respectively. Though they represent an appreciable proportion of the total blood-sugar, the amounts found are fairly constant, ranging between 16 and 31 mg./100 cc. Daily analysis showed narrow fluctuations. Rather remarkable was the finding of fairly constant values after ingestion of commercial glucose which is known to contain an appreciable amount of non-fermentable reducing substances. It was also found that the concentration of these reducing substances in the blood was not affected by insulin.

The above observations apply to the ordinary diabetic. Therefore, if, as Stone suggests, a raised renal threshold is an indication of unfavourable prognosis, it is obviously important to make certain that the hyperglycaemia in suspected cases is also due chiefly to true blood-sugar and not to other reducing substances. As far as the writer is aware, there are no published data on this phase of the subject. Frederick M. Allen [personal communication] states that Richard I. Wagner, while working in the latter's laboratory, made a number of estimations of fermentable and non-fermentable sugar. The sugar which fermented was called "free" and the difference between the free and the total was called "combined." In the cases of raised renal threshold, the values noted were due, chiefly, to the "combined" type.

In this study 58 analyses were made in 19 cases. Blood-sugars were determined both before and after fermentation. All blood samples were obtained in the fasting state, according to the routine of this laboratory. Somogyi's [1927] methods were employed both for purification of yeast and fermentation

of blood, except that for fermentation longer periods and higher temperatures were allowed. For the estimation of the total reducing substances, the Folin-Wu blood-sugar method was used. Briefly, the combined technique was as follows.

Table I. *Showing degrees of hyperglycaemia and amounts of non-fermentable reducing substances in 58 analyses.*

Exp.*	Hospital No.†	Blood-sugar: Before fermentation	Blood-sugar: After fermentation	Blood-sugar: Fermentable	Blood-urea-N mg. per 100 cc.	Exp.*	Hospital No.†	Blood-sugar: Before fermentation	Blood-sugar: After fermentation	Blood-sugar: Fermentable	Blood-urea-N mg. per 100 cc.
1	5996/28	0·825	0·061	0·764	81	30	5312/31	0·270	0·034	0·236	17
2	,,	0·769	0·047	0·722	81	31	2391/29	0·270	0·020	0·250	10
3	6419/29	0·624	0·040	0·584	16	32	,,	0·263	0·037	0·226	10
4	A.A.C.	0·500	0·024	0·476	—	33	431/29	0·263	0·037	0·226	22
5	6249/31	0·500	0·028	0·472	17	34	2391/29	0·263	0·037	0·226	10
6	431/29	0·500	0·040	0·460	22	35	,,	0·256	0·016	0·240	10
7	1935/30	0·454	0·028	0·426	20	36	5312/31	0·250	0·012	0·238	17
8	,,	0·434	0·032	0·402	20	37	6101/25	0·250	0·028	0·222	25
9	,,	0·416	0·031	0·385	20	38	2391/29	0·250	0·028	0·222	10
10	431/29	0·414	0·040	0·374	22	39	,,	0·250	0·010	0·240	10
11	1734/31	0·400	0·027	0·373	14	40	,,	0·244	0·022	0·222	10
12	1935/30	0·400	0·016	0·384	20	41	6443/28	0·244	0·025	0·219	15
13	,,	0·384	0·039	0·345	15	42	,,	0·244	0·016	0·228	15
14	,,	0·384	0·014	0·370	20	43	2391/29	0·238	0·016	0·222	10
15	7135/31	0·370	0·032	0·338	13	44	5312/31	0·233	0·024	0·209	17
16	6443/28	0·344	0·023	0·321	15	45	,,	0·232	0·020	0·212	17
17	431/29	0·333	0·040	0·293	22	46	,,	0·232	0·018	0·214	17
18	6090/30	0·333	0·040	0·293	14	47	431/29	0·232	0·037	0·195	22
19	5312/31	0·333	0·017	0·316	17	48	3535/30	0·232	0·034	0·198	14
20	1935/30	0·319	0·024	0·295	20	49	6443/28	0·227	0·018	0·209	15
21	431/29	0·312	0·030	0·282	22	50	6059/30	0·227	0·047	0·180	10
22	6443/28	0·295	0·042	0·253	15	51	3332/30	0·222	0·040	0·182	20
23	5312/31	0·295	0·025	0·270	17	52	431/29	0·222	0·050	0·172	22
24	,,	0·295	0·033	0·262	17	53	2391/29	0·222	0·027	0·195	10
25	,,	0·285	0·034	0·251	17	54	,,	0·222	0·022	0·200	10
26	2391/29	0·280	0·017	0·263	10	55	5312/31	0·222	0·019	0·203	17
27	5312/31	0·274	0·029	0·245	17	56	2391/29	0·200	0·015	0·185	10
28	,,	0·274	0·025	0·249	17	57	4524/30	0·200	0·024	0·176	16
29	,,	0·270	0·022	0·248	17	58	3953/29	0·200	0·040	0·160	13

* Data arranged in order of degree of hyperglycaemia.
† Hospital numbers do not indicate year of test. They are recorded in order to afford reference to subjects in future study.

The yeast was first purified. A weighed amount was suspended in about 10 parts of water, centrifuged, and the water decanted. This operation was repeated until the supernatant fluid was clear and colourless and the last washing gave no greater reduction with the Folin-Wu copper reagent than did a control consisting of distilled water. This procedure removes the adhering particles of wort and other materials present in yeast, which may be non-fermentable and reduce alkaline copper solutions. As a further precaution, the yeast was suspended in 10 parts of water, and 2 cc. of the suspension were tested for reducing substances by the Folin-Wu process. The yeast was used only when reductions noted were inappreciable and could not be estimated quantitatively. As soon as the yeast was ready for use, a portion of the blood was treated as follows.

One volume of blood was added to 7·5 volumes of a 10 % suspension of yeast[1]. After thorough mixing, the flask was allowed to remain in a water-bath

[1] As Somogyi has shown, it is necessary to allow 7·5 volumes instead of 7 volumes of yeast suspension to correct for the volume occupied by the yeast.

at 37° for 7 min. One volume of 10 % sodium tungstate was then added followed by one volume of 0·66 *N* sulphuric acid. After thorough shaking, the mixture was allowed to stand for 10 min. It was then filtered. The remaining portion of blood was treated in the usual (Folin-Wu) manner. The reducing substances of both filtrates were then estimated simultaneously. In the case of the fermented blood, the necessary precautions were taken for estimating minute amounts of sugar as previously outlined [Folin and Svedberg, 1926]. The combined data are shown in Table I. All values are expressed in terms of percentages and the data are grouped from high to low with respect to the degree of hyperglycaemia.

Discussion of results.

Objections have often been raised against the use of yeast for differentiation between glucose and other sugars. It is however suggested that the various limitations and criticisms of the past do not apply here. The fact that glucose may fail to ferment when present in small quantities applies to urine [Seegen, 1885; von Lippmann, 1904] and to older technical methods, and the fact that yeast may contain, or during fermentation produce, other reducing substances, pentoses, purines, *etc.* [Neuberg, 1910; Mayer, 1913] also need not be considered here, in view of (*a*) the short time allowed for fermentation, (*b*) the technique of purification of yeast and (*c*) the sensitive method for detection of reducing substances. It is therefore assumed that the greater part of fermentation noted in this study was due to glucose.

It will be noted that there was little, if any, relationship between the degree of hyperglycaemia and the amount of non-fermentable reducing substances; though the greatest amount of non-fermentable reducing substances was found with the most marked degree of hyperglycaemia (Exp. 1). However, allowing for the large amount of non-fermentable substance in the latter experiment, there was still a marked hyperglycaemia without glycosuria; the fermentable sugar was 0·764 %. Table II shows the relationship between the degree of hyperglycaemia and the amount of non-fermentable reducing substances more clearly. Maximum, minimum and average values are shown for corresponding degrees of hyperglycaemia.

As food contains many unusable carbohydrate and reducing substances

Table II. *Showing relationship between degree of hyperglycaemia and amount of non-fermentable substances.*

Blood-sugar %	No.	Non-fermentable reducing substances (%)		
		Maximum	Minimum	Average
0·250	23	0·050	0·010	0·026
0·251–0·300	14	0·042	0·016	0·029
0·301–0·400	11	0·040	0·016	0·027
0·401–0·500	7	0·040	0·024	0·032
0·500 +	3	0·061	0·040	0·049

other than glucose, it appeared possible that an observed raised renal threshold in nephritis might be more apparent than real, since nephritis, as is well known, may lead to marked retention of waste products. The cases were therefore divided into two groups, namely, (*a*) those with, and (*b*) those without, nephritis. The criterion of nephritis was the presence of albumin and casts in the urine. As urea determination is a routine in every case of diabetes on admission of the patient to the hospital, the values of this blood-constituent were also made use of. With the exception of one case (No. 5996/28, Exps. 1 and 2), it will be observed (Table I) that the urea contents were practically all within the normal limits for hospital patients[1]. This observation is important, since experiences with many thousands of simultaneous urea and sugar determinations have taught us that hyperglycaemia is usually associated with urea retention only when the latter is fairly marked. It therefore appears from the urea data alone, that the total blood-sugar values noted in many of these cases of raised renal threshold were not due to impaired kidney function. As this view may not be accepted generally, there is the further observation that no relationship was found between albuminuria and the amount of non-fermentable reducing substances in the blood. Thus:

Group	No.	Average amount of non-fermentable reducing substances (mg. per 100 cc.)
Albumin	30	29·5
No albumin	28	28·2

Conclusions.

The raised renal threshold in diabetes is real and not apparent; the hyperglycaemia in these cases is due chiefly to fermentable sugar and not to other reducing substances which are ordinarily found in blood.

In view of the possible finding of as much as 60 mg. of non-fermentable reducing substances per 100 cc. of blood, and in view of the alleged unfavourable prognosis with raised renal threshold, it is suggested that a diagnosis of this condition should not be made without determination of the relative proportion of fermentable and non-fermentable reducing substances.

This work was done with the technical assistance of Miss Mary Beard.

Grateful acknowledgment is due to Mr Julian C. Smith of Montreal, a Governor of this hospital, for his financial assistance in connection with this work.

[1] The urea values recorded are those found on the days of admission of the patients to the hospital, except for Exps. 1 and 2. As none of these patients was suffering acutely from nephritis, it may be assumed that daily fluctuation of values, if it occurred at all, would not affect interpretation of the data.

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CXVI. EVIDENCE OF THE EXISTENCE OF A DIETARY PRINCIPLE STIMULATING GENERAL GROWTH AND LACTATION.

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(*Received May 13th, 1932.*)

THIS work was originally begun to investigate the possible advantages or disadvantages of liver therapy in connection with the nutritional requirements of the growing mammalian organism, and as such the first investigation was undertaken to see if an animal fed on a basal diet containing all the known vitamins and accessory food factors showed any extra growth response when small amounts of fresh liver were fed in addition. There were a few references in the literature which indicated a beneficial effect of feeding whole liver on the growth rate of the animal.

Osborne and Mendel [1926] showed that stock rats fed on a basal ration of caseinogen as protein, starch and salts, yeast and cod-liver oil as a source of vitamins A, B and D, when fed in addition 40 g. of fresh lettuce daily or 1 g. fresh liver daily, showed an accelerated growth response compared with control animals fed on the basal ration alone.

They concluded their paper with the significant remark: "Possibly the acceleration in growth noted is the outcome of stimulation or promotion of activities of cell groups which preside over growth."

Smith and Bing [1929] attempted to improve the growing rate of their stock rats, which received a stock diet consisting of 2 parts whole wheat, 1 part whole dried milk, and some yeast given daily. They found that the addition of fresh lettuce greatly augmented the growth rate of their animals.

Macy, Outhouse, Long and Graham [1927] fed rats on a diet comprising milk powder, whole wheat and salts. Such a ration when supplemented with fresh lettuce daily gave a growth response greater than that obtained by them using other synthetic diets of whole wheat, caseinogen as protein, salts, yeast and cod-liver oil.

More recently Gregg-Smith [1931] has reported that an accelerated growth response may be obtained by the feeding of dried whole liver to rats on a basal diet of starch, cottonseed oil and salts; vitamins were added as yeast and cod-liver oil. The protein in these experiments was given in the form of various tissues which had first been extracted with alcohol till free from fat. No

growth rates are given by the author, but it is stated that the accelerated growth rate obtained is not due to any of the known vitamins.

This evidence, although indirect and, with the exception of Osborne and Mendel's work, somewhat unsatisfactory, since in many cases no carefully controlled experiments were done, pointed nevertheless to a beneficial effect of feeding whole liver on the growth of young rats maintained on synthetic diets.

This influence of fresh liver on the growth rate of the young animal has not only been fully substantiated but now appears to be vastly greater than has been previously supposed. An unidentified substance has been shown to exist in liver capable of intensifying the growth rate to a supernormal degree.

After the completion of the experimental work my attention was drawn to a paper by Daggs [1931]. This worker has investigated the effect of feeding to pregnant bitches on a synthetic diet the following tissues; whole liver, kidney, muscle and eggs. These tissues were fed separately as supplements to the basal rations. He found a much better growth rate of the young puppies and a beneficial influence on the lactation of the mother when liver was fed. The author states that the effect of the liver is due either to a better source of protein or to a more plentiful supply of amino-acids. He, however, suggests the possibility of the existence of a growth-promoting hormone in liver. The results published in the present paper were obtained independently of Dagg's work.

Experimental.

Albino and piebald rats were used. The preliminary experiments were all carried out on piebald rats bred in this laboratory. In the experiments which involved direct feeding of fresh liver to the young animals, the latter were weaned at the age of three weeks and placed on the full synthetic basal diet for a few days before actually starting the experiment.

The basal synthetic diet which has been used throughout the work in the main is:

	Parts by wt.
Rice starch	40
"Light white casein" B.D.H. ...	23
Cane-sugar	17
Palm kernel oil	15
Salt mixture	5
Cod-liver oil	30
Wheat germ	10
Dried yeast	8

This diet has been modified in several experiments which will be mentioned in the text.

The cod-liver oil was incorporated with the basal diet, the latter only being

made up for three days in advance to obviate any possible destruction of vitamin A or other vitamins.

All rats had the synthetic ration *ad lib.* The salt mixture incorporated to the extent of 5 % in the basal ration was as follows:

Sodium chloride	51·8
Magnesium sulphate	79·8
Sodium dihydrogen phosphate ...	104·1
Dipotassium hydrogen phosphate	286
Calcium dihydrogen phosphate	162
Calcium lactate	390
Iron citrate	35·4
Sodium fluoride	0·04
Manganese sulphate	0·2
Potassium iodide	1·00

Although not listed, sufficient copper was present in this salt mixture as an unavoidable constituent.

Experiments have now been completed using both the piebald and albino strain of rat, and the result has been substantially the same in both cases.

Stimulation of growth.

A. *The effect of feeding fresh liver to young rats.*

At the commencement of the experiment the rats were approximately 40 to 50 g. in weight. Ox-liver alone has been used; this was obtained fresh from the abattoir, and was fed at levels of 0·5 to 1 g. of fresh liver *per* rat *per diem*. A smaller dosage of liver has not been given and the question as to the minimum dose to give the maximum effect remains to be determined.

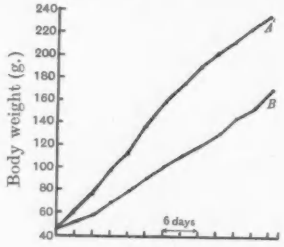

Fig. 1.

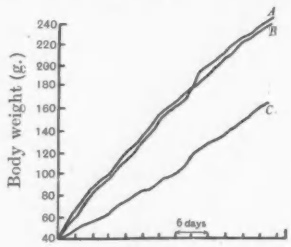

Fig. 2.

Fig. 1. Growth response on feeding 0·5 g. fresh ox-liver as supplement to basal diet to young ♂ rats.
A, liver-fed ♂. *B*, control ♂.

Fig. 2. *A*. Growth response on feeding 0·5 g. liver daily } as supplement to basal diet.
B. Growth response on feeding 1·00 g. liver daily }
C. Control.

Fig. 1 shows the growth response on feeding fresh liver to young male rats. That the effect is not increased by feeding liver at a higher level than 0·5 g. is seen

in Fig. 2. Here it may be pointed out that the possibility exists that the livers themselves may show a seasonal or individual variation and in order to minimise this effect the liver was only obtained in small quantities from the abattoir; by this means fresh liver was always at hand and an average sample of liver tissue was thus fed to an animal over a period of time.

The curves indicate that the growth response to the feeding of ox-liver occurs rapidly. A marked acceleration of growth rate is usually seen for a period of from five to six weeks when compared with the growth rate of the control animals.

The animals fed on liver have been killed and autopsied but no significant increase of adipose tissue which could account for the increase of weight has been detected. The animals appear larger in every respect than their controls of the corresponding age, an increase lying far beyond the range of biological variation.

In the case of the female animal the growth response is much less marked than is the case with the male. Fig. 3 shows the accelerated growth response

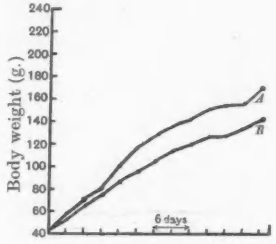

Fig. 3.

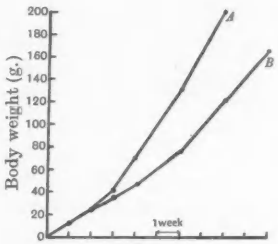

Fig. 4.

Fig. 3. Growth response on feeding 0·5 g. fresh liver as supplement to basal diet to young ♀ rats.
A, liver fed ♀. *B*, control ♀.

Fig. 4. Average growth rate curves of male offspring of parents fed:
A, basal diet + 1·0 g. of liver *per* rat *per diem*.
B, basal diet alone.

of the female animals when fed fresh liver. The average of values used for growth curves lie only just outside the individual variation of the control animals.

It will be noticed from the above results that the direct feeding of fresh liver to young rats led to an immediate response in their growth rates. This impetus to growth was very marked with young animals. The response produced in older animals under such conditions was less marked, indeed the older the animal used, within limits, the less chance was there of obtaining the growth response. Whatever the process was, which had thus been stimulated in the organism by the feeding of raw liver, it was one which, in the young animal, had a much greater effect on the growth rate than in an animal some weeks older.

It therefore seemed possible that feeding fresh liver to male and female animals before mating them might lead to an even greater effect in the case of the offspring. This expectation has been fulfilled.

B. *Transmission from parents.*

Both piebald and albino rats have been used in the breeding experiments. In the mating of parents great care was taken to insure that the various litters were properly distributed in each batch, liver-fed and control. As a rule a batch was made up from two to three litters, brother serving as control to brother, sister to sister. Several batches of rats have now been bred and the results have always been consistent.

The basal diet for these animals was the full synthetic diet containing 10 % wheat germ. In the first experiments whole liver was fed from an early age to both male and female rats; the feeding of the liver to the animals was continued until the end of the lactation period (the male being separated from the female half-way through the gestation period).

The mother received 1 g. of fresh liver daily during the preliminary growth and gestation periods and about double this throughout lactation. The male received 0·5 g. of liver daily. On the day of birth of the litters both control and liver animals were given milk *ad lib.* Weaning occurred in both the control and liver litters after the expiration of 28 days from birth.

The young were allowed no liver and no supplement other than the basal diet mentioned above.

Milk, however, was given them during a period of five weeks after birth. At the age of 21 days the offspring began eating for themselves, and their growth rates were carefully followed.

The weight of the young rat at birth lies between 4·5 and 5·5 g.: no difference could be detected between liver and control litters in this respect. The young were weighed every week, and at the end of 7 and 14 days after birth no significant difference in weight could be detected in the offspring from liver-fed and control-fed parents. During the following week, however, some litters derived from liver-fed parents showed a slight appreciable increase in weight over the control litters, but in the majority of the litters no difference in weight even at the end of 21 days could be detected. From this point, however, the rates and growth curves rapidly diverged, the offspring from liver-fed parents growing much more quickly than the control offspring. The curves in Fig. 4 illustrate these facts.

The time at which the growth response manifests itself in the liver-bred offspring is approximately that at which they begin eating for themselves. The growth curves of the liver-bred offspring are such as might indicate a restricted food supply during the preliminary period of lactation.

Turning to Fig. 5, it is seen that here, as when liver was fed direct to the young animals, the male animal is more greatly affected than the female. It is noteworthy that in the growth curve of the female offspring from liver-

fed parents a distinct break occurs at or about the eighth week; this retardation of growth probably occurs with both groups of offspring but owing to the greater rate of growth of the liver-bred animals it is more marked in their case. This point at which marked retardation occurs corresponds to the onset of puberty. From this stage the growth rate of offspring from liver-fed parents and control parents is practically identical. The female offspring of liver-fed animals thus become larger by a margin equivalent to the accelerated growth obtained during a period from the end of lactation to about the seventh or eighth week.

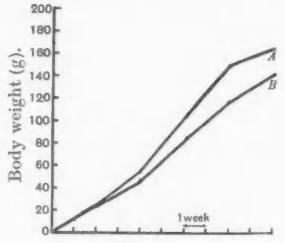

Fig. 5.

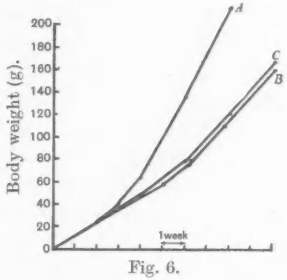

Fig. 6.

Fig. 5. Average growth rate curves of female offspring born to parents fed:
A, 1·00 g. fresh liver *per* rat *per diem* + basal diet.
B, basal diet alone.

Fig. 6. Average growth curves of
A, offspring from parents fed fresh liver;
B, offspring from control animals;
C, second generation offspring from grandparents fed liver.

Final adult weight.

The data at hand indicate a more prolonged growth acceleration in the case of young whose mother has received liver than in the case of direct liver feeding to the young animal. Thus the weight ultimately attained by animals which had been fed liver from an age of 4 weeks throughout their growing period was in the majority of cases only very slightly heavier than that reached by the control animals.

Animals, however, which only received this growth stimulant by transmission from the parent organism, finally attained a weight which ranged from 70–100 g. heavier than that of control animals. Thus the weight generally attained by control animals on the full synthetic diet at an age of approximately twelve months was of the order of 350 to 370 g., whereas the weight of animals receiving the growth stimulant was of the order of 450 to 480 g. Some of these animals are still being kept on the full synthetic diet in order to find if duration of life is affected.

The question as to whether this growth stimulation is prolonged into the second generation has also been studied. The offspring of parent rats which

had been fed on a liver ration as a supplement to the synthetic diet were bred again and the growth rate of their offspring determined. Such second generation rats showed a growth rate practically identical with that given by the control animals (see Fig. 6).

Size of litters.

One further effect of liver feeding, apart from the question of lactation which is dealt with later in this paper, has been noted. This is in connection with the size of the litters born. Table I shows the numbers in the litters from a batch of female rats. This question cannot be definitely answered until large numbers of rats have been studied, since the individual variation in this respect is very great. However, the results at present in hand indicate a larger number of offspring born in the majority of cases to animals which have been fed on liver. The average size of the litter in 20 controls was 7; that in 13 liver-fed cases was 10·3. This means an average increase in the number of young born of 47 %, which is probably too large a figure to be without significance.

In this work no attempt has been made to cut litters down to a maximum of six, and as can be seen from Table I, which is a fair picture of the general

Table I.

Diet of parents	Diet of offspring	Lactation	Number in litter	Average weight of ♂ offspring at 9 weeks g.
Basal + liver	Basal	+	11	246
,,	,,	+	11	255
,,	,,	+	13	235
,,	,,	+	11	280
,,	,,	+	9	270
,,	,,	+	10	295
,,	,,	+	8	230
,,	,,	+	7	260
,,	,,	+	12	240
,,	,,	+	10	270
,,	,,	+	12	275
,,	,,	+	9	245
,,	,,	+	11	265
Basal	Basal	+	8	165
,,	,,	—	7	—
,,	,,	—	6	—
,,	,,	—	6	—
,,	,,	—	10	—
,,	,,	+	8	186
,,	,,	—	6	—
,,	,,	+	7	179
,,	,,	—	9	—
,,	,,	—	7	—
Basal + 8 % dried yeast	Basal	—	7	—
,,	,,	—	5	—
,,	,,	—	8	—
,,	,,	—	8	—
,,	,,	—	4	—
,,	,,	+	6	197
,,	,,	—	9	—
,,	,,	+	9	190
,,	,,	+	5	185
,,	,,	—	6	—

results, despite the higher numbers in the case of the liver-fed litters, the growth rate of the offspring is very much increased after the 4th week. Whether the growth acceleration would have been even greater if the numbers in the individual liver-fed litters had been cut down to the average numbers usually occurring in the control litters remains to be determined. In any case the growth rates of the offspring given above are those from liver-bred litters which contained a larger number of young than the control litters, the advantage, if any, resting with the control litters in this respect.

A larger statistical study is being made with regard to the question of fertility and these results will be reported later.

Period of feeding.

In all the preliminary experiments liver was fed to the parent animals over a long period, from an age of four to five weeks to an age of sixteen to twenty weeks, before the animals were used for breeding. It was therefore important to ascertain whether to obtain this growth response in the offspring it was necessary to feed liver from an early age to the parent animal over a prolonged period. It was conceivable that a greater effect on the offspring might be obtained if liver was fed to the parents during their growing period.

Experiments involving feeding of liver over varying periods of time and at different times in the life history of the animal have been made. Male and female albino and piebald rats were divided into the following groups.

Group (1). Parents which received liver (0·5 g. *per* rat *per diem*) from the age of weaning until the end of the lactation period: mating occurred at the age of sixteen weeks, hence the group received liver during a period of some sixteen weeks.

Group (2). Adult male and female rats which received liver for a period of four to five weeks from the time of mating, the lactation period of the females being thus included.

Group (3). Male and female rats which have been fed on liver, after attaining adult weight, for a period varying from four to five weeks before mating, with a continuation of it during the periods of gestation and lactation.

The data indicate no extra beneficial effect of feeding fresh liver over a prolonged preliminary period before breeding is commenced.

Lactation.

Many workers have reported the failure of mothers to rear and wean their young when fed purified synthetic diets. Some of the earlier work in connection with the failure of lactation is explicable in the light of recent work in that the diets fed were deficient in or had an inadequate supply of the vitamin B complex. Sure [1929] and other workers previously referred to have shown the necessity for an adequate supply of the vitamin B complex if effective lactation is to be obtained. Evans and Burr [1928] found that the diet required as much

as 15 % of brewer's yeast for the supply of B-vitamins if good lactation was to be obtained. Clayton [1930] found, using synthetic rations of milk powder, starch, lard and salts, that lactation failure was largely prevented if the mothers had an abundant supply of the antineuritic vitamin B_1. Evans, working with synthetic diets, maintained that the high "vitamin B" requirements needed for lactation are due entirely to lack of vitamin B_1 in the diet, since normal lactation occurred in diets in which extracts containing vitamin B_1 were added.

The synthetic diet used in these experiments has been shown to be adequate in the vitamin B complex for normal growth. The diet contained all the known vitamin and accessory food factors which have been shown to be necessary for the growth, maintenance and life of the rat. Hence with these considerations in mind it was unlooked for that such a diet should be insufficient for the needs of the lactating female. However, female rats which had been reared and bred on the full synthetic diet for any length of time failed in a large percentage of the cases studied to feed their offspring effectively. This phenomenon was entirely a question of lactation failure. The litters born to such mothers were to all appearances normal in weight and condition, they commonly lived for a period of 1–3 days and eventually died through inanition. In the majority of these cases no attempt was made by the female animal to feed her offspring.

This failure of the lactating power in the case of the control females was common to 70 % of the animals. The litters of the control rats which succeeded in the feeding of their young, were to all appearance in no better condition than those in the mothers of which failure of lactation occurred, yet in the former cases no suspicion of failure to rear and wean the offspring could be detected. A curious feature of these lactation experiments has been the tendency for lactation to proceed in an all or none manner. Only a very small number of cases have been noted in which the parent started to feed her young and subsequently failed before the lactation period elapsed. In the vast majority of cases, if the parent commenced to feed her young, she maintained this throughout lactation.

No such failure of lactation has occurred with those litters whose parents received fresh liver in addition to the full synthetic diet.

The question as to whether the control female animals which failed to feed their young were in any way deficient in vitamin B is answered by the experiments which are more fully dealt with in the next section of this paper.

Manganese consumption.

Orent and McCollum [1931] have recently published work indicating the need of manganese for the proper functioning of the lactating powers of the female. Manganese incorporated in the basal diet at a concentration of 0·005% was found to be sufficient for normal lactation. It was therefore of interest to determine whether a manganese deficiency was the reason for the failure of lactation on the full synthetic diet used in this work.

According to the analytical figures of the manganese contents of various

tissues and plant products given by Reiman and Minot [1920] and more recently by Richards [1930], fresh ox-liver contains 0·170–0·250 mg. manganese per 100 g. fresh weight of tissue. On this basis the liver-fed females were receiving at the maximum only 0·01 mg. more of manganese than the control females *per diem*. The diet used in the early experiments contained, apart from what was present in the wheat embryo, dried yeast and ferric citrate used, 1 mg. manganese per 100 g. On the assumption of a consumption of 10–15 g. of the basal diet *per* rat *per diem* the control animals were receiving a minimum of 0·1 mg. of manganese *per diem*. It seemed hardly conceivable that the addition of 0·002–0·01 mg. to an intake of 0·1 mg. of Mn could remedy the lactation.

Attempts have been made to test this point more completely. The manganese content of the basal diet has been increased to 0·01 % which gives an intake of at least 1·0 mg. *per* rat *per diem* in addition to the manganese occurring in the basal diet. The preliminary results from such an increase of the manganese concentration have shown no beneficial effect. These results will be published in due course.

To eliminate the possibility of there being other unknown essential inorganic constituents in which the diet may have been deficient, fresh liver has been ashed, and the resulting inorganic material incorporated to the extent of 1–5 g. equivalent of fresh liver in the diet and fed to female rats over a period beginning 3–4 weeks prior to mating and continuing throughout the gestation and lactation periods. No beneficial effect was observed.

It is conceivable that the manganese present in fresh liver is in a form more readily utilised than inorganic manganese and the present results may become reconciled to the work of Orent and McCollum in this respect.

Effect of the condition of the mother during lactation.

One further problem has arisen in connection with this lactation study. Turning to those control animals which succeeded in rearing their young, the question arose as to whether the better growth obtained in the young offspring by the feeding of liver to the parents was not due to a more efficient maternal organism, better nutrition and better maternal care during the lactation period. Some evidence with respect to this question has been collected.

In the first place, if this were the case one might expect that it would be during that period when the young were entirely dependent on their mother for nutrition that a difference in the growth rate between the control rats and liver-fed rats would become apparent. One would thus expect on this hypothesis the difference in weight to show itself at the end of that period in which the babies are completely dependent on their parent. The data obtained, however, show at the end of 14–21 days no significant difference in weight between the two groups of offspring.

Secondly, a comparison of the growth of offspring bred on a stock diet used in this laboratory (which is adequate for normal lactation of the rat) with the

growth of offspring bred exclusively on the full synthetic diet used in these experiments supports the view that lactation was fully adequate in this latter case.

Litters bred on the stock diet were transferred to the full synthetic diet at the birth of the young, their growth rates being compared with those of offspring from litters bred entirely on the synthetic diet. No difference in the growth rates of these two groups could be detected. This confirms the observations made earlier that the offspring born to those parent control rats which succeeded in rearing their young on the synthetic diet were normal in all respects.

Nature of the growth principle.

Distinction from known dietary factors.

The first problem which presented itself was the question whether the diet, although it contained adequate amounts of the known vitamins, might yet fail to contain them in sufficient quantity to give the maximum growth response in the young animal. With this viewpoint in mind the basal diet has been altered in various respects and the growth rates on such diets have been studied.

The curves in Fig. 7 indicate the effect of increasing vitamins A and D simultaneously as supplements to the basal diet. These were given as 2–4 extra drops of cod-liver oil *per* rat *per diem*, equivalent to 20–40 mg. of cod-liver oil. It is clearly seen that increase of such vitamins has had no effect on the growth rate of the young animals.

Differentiation from the Coward factor.

Coward, Key and Morgan [1929] showed that rats on a diet consisting of caseinogen, yeast, salts and carbohydrate grew to a weight of approximately 200 g. and ceased growth at this stage, growth only being resumed when certain dietary principles were added, amongst which wheat germ at a level of 5 % of the basal diet was found to be adequate.

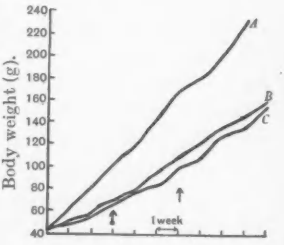

Fig. 7. *A*. Growth rate obtained on full synthetic fresh diet containing 2 % wheat germ + 0·5 g. fresh liver *per* rat *per diem*. *B* and *C*. Growth rate of control animals on basal synthetic diet (2 % wheat germ). ↕ 2 % wheat germ increased to 10 %. ↑ Cod-liver oil increased here.

Fresh ox-liver was also found to have an effect, hence it was natural to suspect that control animals receiving 2 % wheat germ in the basal diet had not sufficient of these principles for maximum growth, and the extra growth response seen in the animals fed liver in addition may have been due to an increased intake of them. Accordingly wheat germ, up to as much as 10 %, has been incorporated into the basal synthetic diet. Fig. 7 shows that increasing the concentration of wheat germ has had no effect on the growth rate.

Miss Coward's work has been repeated and confirmed in its main features, but although additions of wheat germ to diets deficient in this were effective, no effect could be obtained with fresh ox-liver. No explanation of this discrepancy has been obtained except in so far as Dr Guha informed me that he obtained a positive result using liver taken from animals killed during the summer months. Winter liver was used in these experiments and there is the possibility of a seasonal variation in this factor.

In order to eliminate any possible chance of the control animals being deficient in Coward's principle, I have conducted all subsequent experiments on basal synthetic diets containing 8 % of wheat germ. In the results so far recorded the synthetic diet has contained this 8 % wheat germ.

Vitamin B complex.

A third possibility accounting for the increased growth response of the liver-fed animals was a relative deficiency of the vitamin B complex in the basal diet. Many workers have shown in recent years the need for ample supplies of vitamin B for the young animal if good growth is to be obtained.

Macy *et al.* [1927] showed that rats during the lactation period need more vitamin B complex than non-lactating rats. If the female is deprived of vitamin B the young fail to grow and may even die. They find that, if the young are to grow at their maximum rate, three to five times the normal amount of the vitamin B complex is needed by the mother during the lactation period. They define a diet adequate in the vitamin B complex as a basal diet which enables the young to reach a weight of 34–45 g. in weight at the age of twenty-one days.

Sure [1929] has shown that for rats to rear and wean their young a certain minimum amount of the vitamin B complex is required. He also states that this minimum amount is several times greater than the amount required for normal growth of the rat. According to this author the parent has difficulty in passing adequate supplies of B-vitamins to the young during the lactation period unless it is receiving ample amounts of these substances during this time. Under conditions of inadequate vitamin B nutrition the young frequently die of convulsions before the fourteenth day of lactation.

It became necessary therefore to determine whether the active principle in liver yielding the increased growth rate of the offspring of liver-fed mothers and the increased growth rate of the young animal directly fed were not due to an increased supply of vitamins B_1 and B_2. This question has been examined in the following ways.

1. At the age of 4–5 weeks offspring from both control and liver-fed parents were placed on a diet deficient in the vitamin B complex and the time taken to deplete both litters was determined. Now it will be remembered that it is just at this age that the very rapid growth response induced by liver feeding is occurring. Hence if the growth acceleration be due to a better and

higher supply of B-vitamins obtained from the parent animal one might expect the control rats to become depleted far more rapidly than those whose parents had received fresh liver as supplement to the basal diet.

Figs. 8 and 8*a* show, however, that there is no difference in the times at

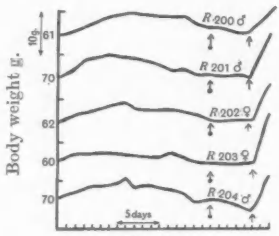

Fig. 8.

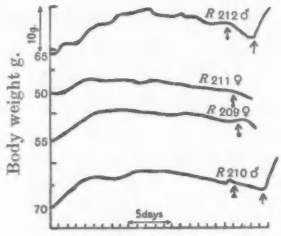

Fig. 8*a*.

Fig. 8. Growth curves of offspring from liver-fed parents, placed on a B-vitamins deficient diet. ↕ vitamin B_2 given. ↑ vitamin B_1 given.

Fig. 8*a*. Growth curves of offspring from control parents placed on a B-vitamins deficient diet. ↕ vitamin B_2 given. ↑ vitamin B_1 given.

which both sets of rats become deficient in B-vitamins. It should be noted that both batches run out of vitamin B_1 before B_2.

That the growth response is not due to vitamin B_1 is also seen from the experiments involving the increase of wheat germ in the basal diet, which had no effect on the growth rate of the young animal.

2. There yet remains the possibility that vitamin B_2 is relatively deficient in the control diet. Some evidence with regard to this question has been obtained by feeding extracts from liver in which vitamin B_2 has or has not been destroyed. Lilly's extract No. 343 and liver extract B.D.H. have both been fed to (*a*) young growing rats, (*b*) parent rats for a period before mating and during the gestation and lactation periods.

Both these extracts contain large amounts of vitamin B_2. The results obtained from such experiments show that both by direct feeding, and by feeding the pregnant female a slightly increased growth rate only is obtained over the control animals. These results are indicated in Fig. 9.

The slightly increased growth rate obtained in these experiments was in all probability occasioned by small amounts of the growth principle adsorbed by the precipitate thrown down by the high alcohol concentrations used in the preparation of the extracts. This will be returned to later.

Further indication that vitamin B_2 is not concerned is given by the preparation of an autoclaved liver extract made according to the method of Guha and Mapson [1931]. This extract contains no vitamin B_1 or B_2. The growth rate given by feeding such an extract was greater than that given by control diets, though not as great as that given by feeding fresh liver.

Such considerations, although partially indirect, indicate that the growth-promoting substance in liver is distinct from vitamins B_1 and B_2.

3. Further direct evidence is at hand. Male and female animals have been fed on the basal synthetic diet containing 7·5 % dried yeast in which the yeast concentration has been raised to 12–16 %. Increase of the vitamin B complex

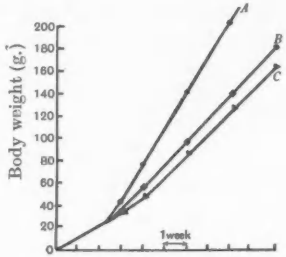

Fig. 9.

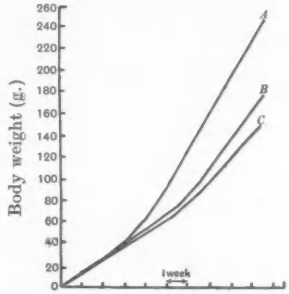

Fig. 10.

Fig. 9. Average growth curves of offspring from parents fed:
A, basal diet and liver.
B, basal diet and extra vitamin B_2 fed as 4 g. liver equivalent of the Lilly ext. no. 343.
C, basal diet alone.

Fig. 10. Average growth curves of offspring from parents fed:
A, basal synthetic and liver.
B, basal synthetic + 8 % dried yeast.
C, basal synthetic.

occasioned by this procedure had only a slight effect in increasing the rate of growth of the offspring (Fig. 10).

The experimental evidence described above negatives the theory that the growth response is due to any of the vitamins of the B-complex.

Protein.

The possibility that the control animals were suffering from any inadequacy of protein or amino-acids seemed unlikely when the high biological value of caseinogen as a protein [*cf.* Hartwell, 1926] was considered. That the active substance is not a protein is seen from the fractionation described below. Whether it is a protein degradation product is uncertain at the present.

Preliminary attempts at fractionation.

The first step undertaken was to ascertain whether absolutely fresh liver was essential in order to obtain this growth acceleration. In these experiments the effect has been tested by the direct feeding of the liver fractions to young animals of an age of approximately 21 days.

Fresh liver was finely minced, brought to a p_H of 4·5–5·0, whereby autolysis is facilitated and bacterial growth inhibited, and allowed to autolyse for 24 hours at 37–40°. After such treatment the autolysate was fed in amounts equivalent to 0·5 g. of fresh liver. Two series of controls have been made in

such experiments: animals fed the equivalent of fresh liver and animals fed the basal ration alone. The growth curves obtained are shown in Fig. 11. It is apparent that the autolysis has had no detrimental effects on the growth stimulating principle. This result was to be anticipated since fresh liver when fed suffers, presumably, a digestion as thorough as that which results from autolysis.

When ox-liver is ashed in a muffle furnace and the resulting ash fed in equivalents of 1–5 g. of the fresh tissue no stimulating effect on the growth of young is produced.

Preparation of active extracts.

Aqueous extracts were made from fresh ox-liver by first mincing the liver and heating the mass to 70° to coagulate the liver-protein and subsequently filtering. The minced material was slightly acidified to a p_H of 5·5–6·0 before heating. Such aqueous extracts when fed in the equivalent of 1·0 g. fresh liver to young rats showed a growth response above that of the control animals.

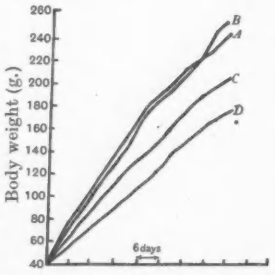

Fig. 11. Growth curves of young male rats fed.

A. Basal synthetic + 1·00 g. fresh liver per diem.
B. Basal synthetic + 1·00 g. equivalent of an autolysed liver extract.
C. Basal synthetic + 1·00 g. equivalent of an aqueous extract.
D. Basal synthetic alone.

An extract made by first autolysing the liver for 24–28 hours and then extracting with water, when fed in the equivalent of 1·0 g. of fresh liver gave a response which was equivalent to that obtained by feeding fresh ox-liver, and which was twice as great as the response obtained by feeding the ordinary aqueous extract. The only apparent difference in the manufacture of the two extracts was a process of autolysis prior to the aqueous extraction in the second case; the p_H of extraction was practically the same in each case. Fig. 11 shows the effects of these extracts on the growth rates of young animals.

This beneficial effect of autolysis prior to extraction is open to two possible explanations:

(1) that the active principle in liver is an integral part of a large molecule and that enzymic change liberates the active smaller molecule, which is then easily extracted;

(2) that the active principle is contained intracellularly in the tissue, and that autolysis by destruction of the cell membrane facilitates its removal.

Fractional separation of the active principle from aqueous liver extracts by means of alcohol has been attempted. Fresh liver was minced, brought to a p_H of 4·5–5·5 with dilute hydrochloric acid, and an equal volume of distilled water added. The temperature of the mass was raised to 70° whereby the liver-protein was coagulated and filtration facilitated. The aqueous extract was concentrated *in vacuo* to a thin syrup. Alcohol was added to make a concentra-

tion of 60 % and the precipitate produced tested on young growing rats was found to be inactive. To the filtrate alcohol was then added to make a concentration of 90 %, and the precipitate was again inactive or only slightly active; the activity remained in the alcohol-soluble fraction. It has been found advantageous in order to obtain the maximum effect to ensure a partial digestion of the liver before making the aqueous extract. This has been effected by, (*a*) autolysis, as previously described; (*b*) digesting the tissue with papain at 60–70° for 10–20 minutes before making the aqueous extract.

Preliminary experiments have also shown that the active substance is extracted from fresh liver by alcohol of a concentration of 90 %[1].

Discussion.

The results recorded in this paper indicate the presence in liver of a substance capable of considerably augmenting the growth rate of the young mammalian organism, when the latter is fed on a full synthetic diet containing all the known essential dietary principles. The growth curves studied, and the general condition and characteristics of the animals which have received such a stimulus to growth also indicate that we are dealing here with a normal physiological process or processes which have been stimulated to an increased activity. The animals have shown no retrogression in weight as frequently occurs when extracts made from certain endocrine organs are injected. Under such conditions a pathological condition of growth is set up, an immense acceleration, frequently followed at the cessation of the injections by a resultant fall in weight. Growth of this type is evidently far from normal in the physiological sense.

It is premature at this stage to say whether the substance active in stimulating the general growth is identical with that stimulating the lactating powers of the female. Further work in this connection is in progress.

The facts described in this paper are suggestive in the light of recent knowledge of a primary stimulation of the anterior pituitary gland. Whilst at this stage any theory advanced as to the physiological cause of these effects must of necessity be merely of a suggestive nature, yet the hypothesis is being borne in mind in this work that we are dealing with some such stimulation of endocrine function.

I have provisionally named the substance promoting growth "physin" until its chemical nature is more clearly defined.

[1] For some of the material used in testing the activity of substances soluble in 90 % alcohol I am indebted to Dr F. H. Carr of British Drug Houses. I am permitted to state that such an extract may be obtained from British Drug Houses, Ltd. by physicians wishing to make clinical experiments on human lactation, *etc.*

SUMMARY.

1. A substance present in fresh ox-liver has been shown to increase very markedly the growth of rats maintained on a diet containing all the hitherto known dietary principles in amount approximately twice the normal.

2. This substance has been shown to have a much more marked effect on the growth rate of the male than the female.

3. A stimulating effect on the growth rate after weaning is transmitted from a parent, which has received the active substance in its diet, to its offspring fed solely on the synthetic diet.

4. It has been demonstrated that liver has also a stimulating effect on lactation; whether the substances stimulating growth and lactation are identical remains to be determined.

5. The average number born to parents fed on liver has been definitely larger than the average number in control litters.

6. The growth-promoting substance has been shown to be distinct from hitherto known accessory food factors.

7. Preliminary investigations into the nature of the active substance are reported.

8. The name "physin" has been tentatively advanced for this substance.

I am indebted to Sir F. G. Hopkins and to Dr L. J. Harris for their kind interest and advice. This work owes much to Miss R. Leader for her untiring assistance with the animals.

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CXVII. THE OCCURRENCE OF SUGAR ALCOHOLS IN MARINE ALGAE.

II. SORBITOL.

By PAUL HAAS AND THOMAS GEORGE HILL.

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(*Received May 18th, 1932.*)

SOME time ago we reported the occurrence of dulcitol in the red seaweed, *Bostrychia scorpioides* [1931]; at that time there was no reason to suspect the presence of any other similar substance, and the detection of a second sugar alcohol, sorbitol, as set forth in this communication, was more or less accidental. The syrupy mother-liquor "S" from which the dulcitol had been precipitated was treated with benzaldehyde in the presence of fuming hydrochloric acid with a view to preparing the benzylidene derivative of dulcitol, it being suspected that the precipitation of the dulcitol had not been complete. To this end 2 g. of the syrup "S" were dissolved in 7 cc. of ice-cold fuming hydrochloric acid and shaken up with 3·5 g. of benzaldehyde; the mixture was left in an ice-chest for 6 hours and then transferred to a glass dish and placed in a vacuum desiccator over caustic potash for 4 days; it was then washed with water until free from acid when 0·6 g. of a buff-coloured solid remained; this was boiled with several successive quantities of alcohol, after which treatment there was obtained 0·2 g. of a white solid. This substance was only sparingly soluble in boiling absolute alcohol but dissolved readily in hot benzene or acetone. Repeated attempts to crystallise it were unsuccessful, and the addition of light petroleum or alcohol to an acetone or alcohol solution invariably led to the formation of an amorphous precipitate the melting-point of which could not be raised above 180–182°. The melting-point quoted in the literature for the tribenzylidene derivative of dulcitol is 215–220°, while that of the corresponding derivative of sorbitol, which is described as amorphous, is variously given as 172–175° [Meunier, 1891] and 179–184° [Davis, Slater and Smith, 1926]. The former author regarded the product as a mixture of the mono- and di-benzylidene derivatives as evidenced by his combustion figures which were consistently about 2 % low for the carbon. Analysis of our material, however, gave figures suggesting rather that it was a mixture of a tribenzylidene derivative with some of the di-derivative:

	C %	H %
Found ...	71·37	5·88
Calc. for $C_{27}H_{26}O_6$	72·65	5·83
„ $C_{20}H_{22}O_6$	67·04	6·14

As some further evidence for the identification of sorbitol seemed necessary, 7 g. of the syrup "S" were heated with 10 g. of acetic anhydride and 1 g. of pyridine for one hour over a sand-bath [Tutin, 1925]; after removal of the excess of acetic anhydride and washing there remained a light yellow syrup which rapidly set to a mass of crystals which, after pressing on a porous tile, weighed 0·5 g.; they were recrystallised from a mixture of ethyl acetate and light petroleum from which they separated in well-formed, flat, elongated prisms melting at 98–99°, the melting-point given for hexa-acetylsorbitol by Tutin being 99°, while that of hexa-acetyldulcitol is 171°. Furthermore the substance when mixed with an authentic sample of hexa-acetylsorbitol still melted at 98–99°.

From these data there could no longer be any doubt that the syrup "S" actually contained sorbitol.

As a further confirmation, however, some of the benzylidene derivative above described was hydrolysed with dilute sulphuric acid and steam-distilled to remove benzaldehyde; after neutralising the sulphuric acid with baryta and evaporating there remained a syrup which, treated with alcohol, gave, after some time, crystals of sorbitol.

Whilst no claim to an accurate estimation can be made, the evidence from the amount of crude material obtained from the syrup of *Bostrychia* indicates that this material contained approximately 8·3 % of dulcitol and 13·6 % of sorbitol.

Comment has already been made on the fact that the only sugar alcohol previously recorded as occurring in marine algae was mannitol [Haas and Hill, 1931]. With the establishment of the presence of both dulcitol and sorbitol in *Bostrychia*, all three alcohols are now known to occur in this group of plants, but we have found no evidence of the presence of dulcitol or sorbitol in any other of the Rhodophyceae of which we have examined the following: *Batrachospermum moniliforme*, *Chylocladia articulata*[1], *Corallina officinalis*, *Cystoclonium purpureum*, *Gigartina stellata*, *Laurencia pinnatifida*, *Polysiphonia fastigiata*, *Rhodymenia palmata*.

It is moreover worthy of note that outside the family of the Rosaceae only very few occurrences of sorbitol have been recorded.

The reaction between sugar alcohols and benzaldehyde.

The problem here presented of detecting both dulcitol and sorbitol in the same mixture was made the occasion for an investigation of the relative ease with which the three sugar alcohols react with benzaldehyde. For this purpose three equal quantities of mannitol, sorbitol and dulcitol were treated simultaneously with quantities of fuming hydrochloric acid and benzaldehyde; on shaking the resulting mixtures mannitol and sorbitol set almost immediately to a solid mass while the solution containing the dulcitol remained liquid. After

[1] Kindly supplied by Miss M. Parke of Liverpool University.

standing for five hours in the ice-chest the products were washed under identical conditions; the weights of crude material obtained from the three experiments corresponded to approximately 100 and 95·6 % yields from mannitol and sorbitol respectively, and only 38 % from dulcitol.

As an extension of this observation a mixture of equal parts of dulcitol and sorbitol was treated with fuming hydrochloric acid and benzaldehyde as above: it was found that the weight of crude product corresponded to a yield of about 60 % of the amount which should have been produced on the assumption that both alcohols had been converted into tribenzylidene derivatives, but the product consisted for the most part of the sorbitol derivative since approximately 50 % of the dulcitol was recovered unchanged.

From the experience gained by the above experiment it is clear that where dulcitol and sorbitol occur together in the same solution they can be reasonably well separated by precipitating the dulcitol in crystalline form by the addition of alcohol and by recovering the sorbitol from the mother-liquor by conversion into its benzylidene derivative.

The adsorption of sugar alcohols by basic lead acetate.

It is an established fact that soluble carbohydrates tend to be removed from solution by adsorption upon basic lead acetate precipitates if the latter are left in contact with the solution for some hours. Evidence has now been obtained that the same holds for dulcitol and sorbitol.

A 60 % alcoholic extract of *Bostrychia scorpioides*, after distilling off the alcohol under reduced pressure, was precipitated with excess of basic lead acetate and set aside overnight. Next morning the precipitate was filtered and washed very thoroughly by repeated grinding under hot water and filtering. The resulting precipitate was decomposed with hydrogen sulphide and the filtrate from the lead sulphide neutralised with baryta and evaporated to small bulk under reduced pressure; it was then treated with twice its volume of alcohol, filtered and evaporated to a syrup; on cooling typical crystals of dulcitol separated (M.P. after recrystallisation 185–186°), showing that, in spite of the thorough washing to which the lead precipitate had been subjected, some of the dulcitol had adhered to it.

The filtrate from the dulcitol crystals was shown to contain sorbitol by conversion into the benzylidene derivative. In addition this same solution was shown to contain an ethereal sulphate since, on hydrolysis with hydrochloric acid, it gave a heavy precipitate of barium sulphate due to the interaction between the barium acetate present in the solution with sulphuric acid liberated as a result of the hydrolysis. In this respect, therefore, *Bostrychia* resembles other red algae.

SUMMARY.

1. *Bostrychia scorpioides* contains the sugar alcohol sorbitol as well as dulcitol.

2. In common with other Rhodophyceae, it also contains an ethereal sulphate complex.

3. The reaction between sugar alcohols and benzaldehyde is commented on.

4. The marked adsorption of dulcitol and sorbitol upon precipitates with basic lead acetate is noted.

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CXVIII. THE METABOLISM OF CARBOHYDRATE AFTER STARVATION.

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In a recent paper Dann and Chambers [1930] have reopened the question of the intolerance to carbohydrate which follows a period of starvation. The phenomenon has been examined by one of us [Goldblatt, 1925] and it was found that, after a period of some 40 hours' complete starvation in human subjects, administration of a dose of glucose, normally easily tolerated, was followed by high and delayed blood-sugar curves, glycosuria and a rise in R.Q. very much less than that occurring in ordinary post-absorptive conditions. The possible causes of this have been discussed by various authors. Thus, Brailsford Robertson [1923] suggested that the period of starvation was associated with a diminished secretion of insulin, so that the intensive administration of glucose found the mechanism for control of blood-sugar temporarily ineffective. Other workers have taken the view that it is due to the appearance of ketosis or to the tendency to acidosis. The fact, however, that the phenomenon is easily demonstrable in animals which do not develop a ketosis during starvation seems to rule out effectively its attribution to this cause. To show that the tendency to acidosis might be responsible, attempts have been made to readjust the disturbed mechanism by the administration of alkalis.

Dann and Chambers [1930], using dogs starved for about 3 weeks, have found well marked intolerance to glucose as manifested by a failure of the R.Q. to rise after the administration of sugar by more than 0·06. On the day following such an experiment a further test with glucose produced marked rises in R.Q. These results are similar to those found by one of us in human subjects, when it was shown that the administration of 50 g. of sugar readjusted the disturbed mechanism so that a subsequent dose gave a normal tolerance curve. Dann and Chambers have further made the interesting observation that complete readjustment of carbohydrate metabolism cannot be obtained even when 20 units of insulin are injected in addition to the ingestion of glucose. They conclude therefore that starvation-intolerance to carbohydrate may be associated with some as yet unknown change in the tissues, following upon disuse of the sugar-oxidising mechanism. In one of their experiments they found that after a 19-day fast, ingestion of 50 g.

[1] In receipt of a grant from the Medical Research Council.

glucose and injection of 20 units insulin, the blood-sugar 4½ hours later was 0·220 %, whilst on the next day in the same animal the blood-sugar in similar circumstances was 0·116 %, and on the succeeding day 0·087 %. They show clearly that the insulin response became progressively better as the metabolism of the animal was readjusted by the carbohydrate. *Pari passu* with these findings the utilisation of sugar as calculated from respiratory data increased steadily, the values being 2·13, 4·34, and 4·50 g. glucose per hour. Although these authors made no determinations of alkali reserve of the blood, there can be no doubt that their animals were in a state of more or less compensated acidosis. The relative ineffectiveness of the insulin injection may be referable to this acidosis and is comparable with the clinical finding that severely acidotic diabetic subjects are resistant to the action of the hormone [Koehler, 1926]. It has further been found by several workers that administration of alkali to such severely acidotic subjects brings about a better response to insulin. It is therefore not necessary, without further knowledge, to attribute this failure of the starved dog to respond adequately to insulin to specific tissue changes, changes in tissue reaction being perhaps sufficient to account for this observation.

Using dogs which had been starved for from 3 to 7 days, Hines *et al.* [1929] found that intravenous injection of 4 g. glucose per kg. per hour produced blood-sugar values and glycosuria which indicated diminished tolerance, but the R.Q. was high and the heat production rose by 50 %. These results are entirely out of harmony with those of Dann and Chambers [1930] and of Goldblatt [1925]. It seems improbable that the discrepancies can be explained by the shorter period of starvation of the dogs since definite intolerance had been established, unless we suppose that it is possible to dissociate the glycogen-storing power of the liver from the oxidising function in the peripheral tissues. Such a dissociation is met with in the human subject in those cases of glycosuria without ketosis in which a dose of glucose produces a considerable oxidation response. In view of the differences of opinion on these matters we present the following experiments.

Experimental methods.

Blood-sugar was determined by MacLean's method, respiratory metabolism by the Douglas and Haldane technique, urinary nitrogen by Kjeldahl's, and urinary sugar by Bertrand's method. Acetone substances were estimated by a method described by one of us [Goldblatt, 1925]. Plasma-bicarbonate was determined by Van Slyke's method after equilibration with normal alveolar air; plasma-inorganic phosphorus by Briggs's modification of the Bell-Doisy method; blood-lactic acid by Friedemann, Cotonio and Shaffer's method after precipitation with $CuSO_4$–$Ca(OH)_2$ in the Folin-Wu tungstic acid filtrate.

Exp. 1. *The effect of starvation on the carbohydrate tolerance of normal rabbits.* The rabbit is an animal which in our experience does not produce ketones on starvation. The demonstration of starvation intolerance in these animals

would therefore dispose effectively of the view that ketosis is a necessary accompaniment of this phenomenon. Three rabbits (3 kg.) were taken and blood-sugar curves obtained after the administration of 4 g. glucose in 20 cc. water by stomach tube. The animals had been accustomed to handling and all gave low initial blood-sugar values. During starvation they were allowed water *ad lib.*

Table I. *Blood-sugar values (mg. per 100 cc.) were obtained ½-hourly after 4 g. glucose* per os.

Diet preceding starvation period—bran, oats, cabbage.

	Rabbit 1	Rabbit 2	Rabbit 3	Average blood-sugar	Mg. increase above fasting value
		(*a*) After 27 hours' starvation.			
Fasting	104	109	104	106	—
	166	162	162	163	57
	129	137	159	142	36
	113	106	159	126	20
2½ hours	113	109	129	117	11
		(*b*) After 72 hours' starvation.			
Fasting	65	81	102	83	—
	83	175	115	124	41
	115	199	143	152	69
	106	187	137	143	60
	129	206	171	169	86
2½ hours	106	139	191	145	62
	111	92	125	109	26
	92	—	—	—	—
(*c*) After 72 hours' starvation. 1 g. $NaHCO_3$ administered with the glucose.					
Fasting	69	90	109	89	—
	100	171	191	154	65
	144	201	213	186	97
	83	154	129	122	33
	156	175	197	143	54
2½ hours	135	152	141	143	54
	125	115	120	120	31

The last column of Table I shows the definitely diminished rate of readjustment of blood-sugar after 3 days' starvation. Further it will be seen that the addition of 1 g. $NaHCO_3$ to the glucose was not followed by any improvement in tolerance. Such a dose of alkali is sufficient to produce a neutral urine in rabbits of this size. Du Vigneaud and Karr [1925] found that the administration of 1 g. $NaHCO_3$ daily to rabbits during a 4-day fast prevents the abnormal response to sugar. Their results appear to resolve the problem into a carbohydrate intolerance due to a diminution in available base. Our results do not bear this out nor did our animals show any tendency to acidosis, but we do not assert that the intolerance is unrelated to acidosis. The matter with which, however, we are immediately concerned is that starvation intolerance is not necessarily accompanied by ketosis. Eisner [1926], confirming the findings of others in the matter of the abnormal blood-sugar curve after starvation, also found that during the 3 hours following the administration of 35 g. glucose to rabbits starved for 10–11 days the deposition of glycogen in the liver and muscles was very poor compared with that found in ordinary

conditions. Twenty-four hours after the sugar was given the glycogen deposition was normal. These results seem to leave little doubt that starvation leads to a diminished power to store glycogen in the tissues. The later adequate deposition of glycogen is simply the expression of the readjustment to normal which carbohydrate is able to effect. In an experiment [Goldblatt, 1925] with starving human subjects it was found that readjustment to normal carbohydrate metabolism was established $2\frac{1}{2}$ hours after a dose of 50 g. fructose even though at this time ketosis was again appearing.

The remaining experiments in this paper were performed on two normal human subjects, whose metabolism had been determined on several occasions and found to remain steady during many hours of experiment. Starvation was undergone during full working days in the laboratories and the wards. During the experimental investigations of the results of starvation the subject was kept at complete rest in bed. Collections of blood and urine were made with the minimum of disturbance, the subject being well used to the procedure.

Experiments with Subject I. (*Normal adult man, 150 lbs. in wt.*)

For comparative purposes it will be necessary to give the blood-sugar curves obtained with this subject after administration of 50 g. glucose in ordinary conditions.

Table II. *Blood-sugar curves of Subject I.*

3 hours after a light meal		16 hours after food	
Minutes after glucose	Blood-sugar mg. per 100 cc.	Minutes after glucose	Blood-sugar mg. per 100 cc.
0	122	0	102
5	129	30	150
25	159	60	113
45	150	90	91
65	125	120	75
85	117		

Glycosuria never occurred in these conditions. It will be seen that complete readjustment to fasting blood-sugar levels is reached in about 90 minutes.

Now, if the intolerance to carbohydrate which occurs after starvation is due to acidosis or deficiency of base, it should be possible to demonstrate this by some of the simple tests at our disposal. In the next experiment we used the principle of the very simple and very useful Sellard's test.

Exp. 2. Subject I underwent a period of 37 hours' starvation. After a period of rest 5 g. $NaHCO_3$ in 50 cc. water were taken. About 30 minutes later 50 g. glucose in 100 cc. water were ingested and the observations recorded in Table III were made.

At 3.20 p.m. the urine was again acid to litmus.

The delayed return to fasting blood-sugar levels and the glycosuria are sufficient indices of the disturbed tolerance to carbohydrate. As in the earlier experiment [Goldblatt, 1925], the ketosis disappeared in about an hour after the glucose was taken. The rise in ketone excretion (from about 5 to

Table III.

Time	Blood-sugar mg. per 100 cc.	Urine: Vol. cc.	Urine: Glucose mg.	Urine: Titratable acidity cc. $N/10$ per hour	Urine: p_H	Urine: Total ketones as mg. acetone per hour
10.30 a.m.	83	—	—	—	—	—
11.15 „	—	26·5	0	12·37	5·5	4·69
5 g. $NaHCO_3$ taken.						
11.45 a.m.	81	17·0	0	12·75	5·5	5·16
50 g. glucose taken.						
12.15 p.m.	135	17·0	0	15·30	5·5	10·60
12.45 „	166	17·0	0	18·70	5·5	2·58
1.15 „	141	18·5	130	11·10	6·5	Trace
1.45 „	115	22·0	0	6·60	8·0	0
2.15 „	104	19·0	0	3·80	8·5	0
2.45 „	83	14·0	0	2·80	8·3	0

10 mg. per hour) is the invariable accompaniment of alkali ingestion during ketosis. The combination of bicarbonate and glucose produced a marked shift in urinary reaction to the alkaline side but the defective carbohydrate control was not corrected. Taking into consideration, however, that at the time of ingestion of the sugar the excretion of acid was actually greater than at the outset and further that the sugar alone is able to produce a reduction in acidity, we must conclude that 5 g. $NaHCO_3$ is insufficient to neutralise the incipient acidosis. This will be clearer in the next experiment.

Exp. 3. In this experiment two doses of bicarbonate were given and the glucose was taken when the urine was definitely alkaline (to litmus). Subject I underwent a period of 39 hours' starvation before the beginning of the observations recorded in Table IV.

Table IV.

Time	Blood-sugar mg. per 100 cc.	Plasma-bicarbonate cc. CO_2 per 100 cc.	Urine: Vol. cc.	Urine: Glucose mg.	Urine: Titratable acidity cc. $N/10$ per hour	Urine: p_H	Urine: Ketones as mg. acetone per hour
9.00 a.m.	(overnight collection)		227	0	13·82	5·0	8·01
10.30 „	92	—	—	—	—	—	—
11.00 „	—	64·3	41	0	11·28	5·0	11·77
5 g. $NaHCO_3$ taken.							
11.30 „	92	—	—	—	—	—	—
12.04 p.m.	—	69·0	29	0	10·15	6·5	19·92
12.45 „	75	—	—	—	—	—	—
1.04 „	—	—	34	0	9·35	6·8	50·94
1.34 „	—	—	12	0	12·00	5·5	12·45
5 g. $NaHCO_3$ taken.							
1.45 „	87	—	—	—	—	—	—
2.30 „	—	—	21·5	0	9·21	7·5	38·14
2.55 „	87	86·5	—	—	—	—	—
3.15 „	—	—	28·5	0	2·85	8·0	63·53
3.25 „	50 g. glucose taken.						
3.55 „	131	—	—	—	—	—	—
4.00 „	—	—	19	0	4·43	8·0	38·32
4.25 „	168	—	12	0	11·76	—	10·40
4.55 „	222	—	21	462	7·88	—	Trace
5.25 „	162	—	—	—	—	—	—
5.33 „	—	—	23	97	7·66	7·5	0

It is clear from these results that 5 g. $NaHCO_3$ were sufficient to bring the urinary reaction from p_H 5 to p_H 6·8, *i.e.* just short of neutrality, in about 2 hours. The second dose of bicarbonate, given 30 minutes after the effect of the first dose had passed off, produced an alkaline urine in $1\frac{3}{4}$ hours, at which point the glucose was taken. Throughout the succeeding 2 hours the urine was at p_H 7·5–8. In spite of this a very abnormal tolerance curve was obtained and 0·56 g. sugar appeared in the urine. It is further to be observed that even at the beginning of the experiment the bicarbonate reserve of the plasma (equilibrated with normal alveolar air) indicated full compensation of any acidotic tendency. (The value 86·5 seems very high with these doses of $NaHCO_3$.) The very large increases in ketone excretion following each dose of glucose are also noteworthy.

It seems justifiable, therefore, to conclude thus far that, whilst judged from Sellard's test there is a slight deficiency in available base, there is full compensation as far as the blood is concerned, that is to say that the production of abnormal acids after these periods of starvation is not more rapid than can be effectively buffered in the blood. This does not eliminate the possibility of local changes in the tissues. But when after the second dose of bicarbonate an alkaline urine is produced we may fairly certainly assume that tissue buffering has also been attained. When, therefore, we obtain definite evidence that the diminished tolerance to carbohydrate still persists we are forced to the view that it is not primarily due to lack of available base.

The increased elimination of ketones after alkali administration is well known and is associated with a great depression and even disappearance of ammonia formation. Even with the relatively small doses we used there occurred a very large increase in ketonuria accompanied by very marked falls in titratable acidity.

It has been demonstrated by Gamble *et al.* [1923] that during starvation (3–15 days) there was no depletion of fixed base in the plasma, although the plasma-bicarbonate fell from 66 vols./100 cc. before the fast to 41 vols. on the third day. In our experiments, however, the periods of starvation were much shorter, so that no depletion either of fixed base or of plasma-bicarbonate was demonstrable. Since in the experiments of Gamble *et al.* [1923] the plasma-bicarbonate fell without diminution of fixed base, it is probable that there was a transfer of the latter from the HCO_3 ion to the anions of the ketonic acids.

Hirsch [1927] investigated the effect of sodium acetate on the loss of carbohydrate tolerance of a normal man after 38 hours' starvation. 50 g. glucose given after the period of fasting raised the blood-sugar from an initial value of 84 mg./100 cc. to 169 mg./100 cc. in 98 minutes, and after 216 minutes the blood-sugar was still 93 mg. Administration of 2 g. sodium acetate to the same subject after another similar period of starvation led to the production of a neutral urine, and 50 g. glucose then raised the blood-sugar from 85 mg./100 cc. to 153 mg./100 cc. in 186 minutes and after 245 minutes the value was 70 mg./100 cc. Although Hirsch regards these values as supporting the view that the sodium acetate improved the tolerance, it is difficult to

reconcile this with a blood-sugar of 153 mg./100 cc. 186 minutes after giving the sugar. The value of 70 mg./100 cc. some 4 hours after giving the sugar, however, suggests a better utilisation. The R.Q. in the experiment with acetate rose from 0·756 to 0·854, whilst without acetate it rose from 0·756 to 0·808: the increase in oxygen utilisation was, however, rather better in the experiment without alkali. With starving rabbits (3 days) and dogs (4 days) this author obtained lower blood-sugar curves when alkali was administered.

To examine further whether starvation-intolerance to carbohydrate after relatively short fasts was associated with changes in acid-base balance we studied the urinary ammonia after various periods of starvation.

Exp. 4. This experiment was divided into two parts.

(*a*) Subject I starved for 18 hours, during the last 6 hours of which the urine was collected hourly. Total nitrogen, ammonia, and ketones were estimated (Table V).

Table V.

Time without food (hours)	Urine: Vol. cc.	Urine: Total N g. per hour	Urine: Ammonia-N mg. per hour	Urine: Ketones mg. per hour (acetone)	NH_3/N
13	75	0·615	28·40	4·51	1/20·9
14	75	0·599	30·98	5·97	1/19·3
15	70	0·524	30·87	6·11	1/17·0
16	55	0·451	30·03	5·19	1/15·0
17	60	0·440	26·88	6·65	1/16·4
18	70	0·473	26·95	3·46	1/17·6

Remembering that 1 g. ammonia is capable of combining with 6 mg. acetoacetic acid (3·4 mg. acetone) we see that the relatively large excretion of NH_3 is here a mechanism for dealing with acids other than the ketone bodies. It is also seen that as the period of starvation increases the fraction of the degradation products of protein which is transformed to ammonia tends to increase. For our present purpose the important point is that if at any of the times given in Table V a sugar tolerance test be performed a so-called normal curve will be obtained. In the second part of this experiment similar observations were made from the 40th to the 45th hour of starvation, 50 g. glucose being taken after determination of basal values.

Table VI.

Time	Urine: Vol. cc.	Urine: Total N g. per hour	Urine: NH_3-N mg. per hour	Urine: Ketones mg., per hour (acetone)	NH_3/N
11.30	—	—	—	—	1/23·5
12.30	50	0·499	21·00	3·44	1/23·8
1.30	40	0·376	20·16	5·32	1/18·6
1.40	50 g. glucose in 100 cc. water.				
2.33	25	0·412	23·45	4·70	1/17·6
3.00	12	0·427	27·89	0·00	1/15·3
4.00	42·5	0·557	29·16	2·82	1/19·8
5.00	97	0·543	23·09	3·73	1/23·6
5.15	9·5	—	—	—	—
5.45	11	0·255	11·55	3·97	1/22·1

(*b*) Subject I underwent a period of 40 hours' starvation prior to the beginning of the experiment in Table VI.

The degree of intolerance to sugar which developed in the experiment produced glycosuria and the half-hourly blood-sugar values were:

Time in hours	0	½	1	1½	2	2½	3	3½
Blood-sugar, mg. per 100 cc.	74	141	166	141	129	117	120	87

Glycosuria occurred between ½ and 1½ hours from taking the sugar, so that we may be certain that there was a temporary elevation above the threshold for glucose. The ammonia excretion was lower to start with than in Table V, so that from the point of view of acidosis we had no reason to suspect the existence of a low tolerance for sugar. The absolute amount of ammonia excreted rose steadily and began to fall when the fall in blood-sugar became marked, until, when the latter reached normal fasting values, the ammonia had fallen to 11·55 mg. per hour, and evidence of protein-sparing appeared in the much lowered nitrogen excretion.

We are thus constrained to doubt whether starvation intolerance to carbohydrate is primarily due to acid intoxication. It is well known that marked disturbances in acid-base equilibrium are associated with loss of tolerance to sugar, and so there can be no doubt that starvation of long enough duration to produce changes in tissue- and blood-reactions will in virtue of these changes lead to greater loss of tolerance. But that other and more subtle factors may be involved is indicated by our demonstration that the loss of tolerance may occur without associated acidosis, and that it is not completely corrected by such doses of alkali as are sufficient to produce neutral or alkaline urine. The fact that many observers have insisted that the lowered sugar tolerance in man begins to manifest itself when ketosis appears is not evidence that either ketosis or acidosis bring it about. Ketosis is an expression of a preponderance of fat metabolism, so that a possible view to take is that diminished tolerance to sugar after starvation is due to an inability of the liver cells to readjust rapidly from a preponderance of fat to a plethora of carbohydrate. It has been clearly shown by Kohn *et al.* [1927] and Ellis [1931] that during a period on a ketogenic diet children develop very great loss of tolerance to sugar but that following the ketogenic régime there is a period of very greatly increased tolerance before the return to normal. Thalhimar (quoted by Kohn *et al.*) tentatively suggests that over-secretion of insulin may be the cause of the increased tolerance in the *post*-ketosis period. There is, however, no direct evidence that during the ketosis induced either by ketogenic diet or by cyclical vomiting or by starvation there is any deficiency or excess of insulin. The experiments of Dann and Chambers [1930] suggest strongly that other factors than deficiency in insulin are concerned in the diminished sugar tolerance in starvation.

In the following experiments detailed examinations were made of the partition of heat production between the various nutritional constituents during starvation periods sufficient to induce a lowered tolerance to carbo-

hydrate. For this purpose we used Subject II. Comparisons were made between the metabolic state of this subject 16 and 39 hours after food, and the effect on metabolism of the ingestion of 50 g. glucose. Calculations of the partition of energy between fat, carbohydrate and protein were made by means of the Zuntz-Schumburg table as modified by Lusk, the ordinary corrections for nitrogen being made. In some cases estimations of the capillary and venous blood-sugar of the same arm were made.

Differences between capillary and venous blood-sugar are generally interpreted as evidence of sugar utilisation by the muscles, the fact that diabetics show very little difference between the two values providing corroboration of this view. But capillary-venous differences in blood-sugar may also indicate deposition of glycogen in the muscles, particularly if the latter have been depleted of their normal content by starvation or other means. Thus, in starvation, capillary-venous differences following glucose administration may be as marked or even more marked than in normal condition without this necessarily indicating as good or better oxidation of sugar. This will be shown in the experiments which follow in which great diminution in oxidation of carbohydrate was accompanied by capillary-venous differences in blood-sugar as high as occur without starvation.

The association of phosphorus with sugar metabolism led us to estimate plasma-inorganic phosphorus. The administration of glucose to a subject able to metabolise sugar normally is followed sometimes by a fall in plasma-inorganic phosphorus, but this returns to normal levels in an hour or two [Hartmann and Bolliger, 1925; McCullagh, 1931]. Insulin is also able to produce definite falls in plasma-inorganic phosphorus. Mild diabetic cases, however, show in similar circumstances a fall in phosphorus but there is a much slower return to normal values. Severe diabetics give no fall at all. In our experience the diminution in plasma-inorganic phosphorus is not always demonstrable after the ingestion of sugar, which may perhaps be due to the transitoriness of the change. Simultaneously with the fall in plasma-inorganic phosphorus there occurs a marked fall in urinary excretion of phosphate, normal excretion returning with the return to normal plasma values. If we regard the amount of circulating phosphate in the starving organism in the early stages as the resultant of the processes of formation and breakdown of hexosephosphoric esters and the excretion of urinary phosphate, then it appears probable that in the early stages of carbohydrate utilisation there is preponderance of synthesis of esters. In view of the fact that in the normal subject the fall in plasma-inorganic phosphorus is only of short duration whereas in the mild diabetic it is more prolonged, it is possible that the formation of phosphoric esters (regarded as intermediate steps in carbohydrate metabolism) is unimpaired in the mild diabetic but his oxidative capacity is deficient, so that the ester stage is only slowly passed through. In the very severe diabetic the fact that the blood-inorganic phosphorus does not fall at all is explicable on the view that both mechanisms are at fault so that he

neither esterfies his carbohydrate nor oxidises it. As will be seen later on, findings with Subject II seem to point to a response of plasma-inorganic phosphorus of the type associated with mild diabetes. Blood-lactic acid was estimated as an index of emotional and muscular rest and remained constant throughout.

Exp. 5. *Metabolism of 50 g. glucose by Subject II, after 16 hours' fast* (Table VII).

Table VII.

Blood analysis.

Time after glucose mins.	Blood-sugar mg. per 100 cc. Capillary	Blood-sugar mg. per 100 cc. Venous	Plasma-CO_2 combining power vols. %	Plasma-inorganic P mg. per 100 cc.	Lactic acid mg. per 100 cc. whole blood
0	111	109 (3)	57·6	3·13	13·2
33	109	109 (5)	57·6	3·26	13·8
66	148	134 (2)	59·5	3·43	14·4
133	156	117 (5)	58·6	3·45	13·2

Venous blood taken ( ) minutes after capillary blood.

Respiratory metabolism.

Time after glucose mins.	Urinary nitrogen mg. per min.	Non-protein R.Q.	O_2 cc. per min.	g. metabolised per hour Protein	Fat	Carbohydrate
Basal	11·56	0·81	258	4·34	3·34	4·76
Basal	12·70	0·88	248	4·76	2·25	7·39
28	9·70	0·78	268	3·64	4·78	3·64
58	10·51	0·93	244	3·94	1·39	10·08
93	10·51	0·93	285	3·94	1·70	12·31
130	10·51	1·04	270	3·94	0	15·72

The important points in this experiment are the following.

(1) There was some delay in the rise of blood-sugar; the value 156 (capillary) which appears rather late for a normal subject is referable probably to slow absorption[1].

(2) The plasma-CO_2 was normal and remained unchanged.

(3) The inorganic phosphorus in the plasma rose definitely during the 2 hours of the experiment.

(4) The respiratory metabolism gave unequivocal evidence of active oxidation of carbohydrate. Whilst the protein metabolism per unit time remained sensibly constant, the fat catabolism gradually diminished until when the non-protein R.Q. was 1·04 the usual assumptions led to the overwhelming preponderance of carbohydrate in the non-protein catabolism.

The attainment of a capillary-venous blood-sugar difference of 39 mg. per 100 cc. at the same time as an R.Q. of 1·04 may be regarded as additional evidence of active disposal of carbohydrate. Taking this as a typical reaction of Subject II to 50 g. glucose, we now proceed to give the results obtained after two periods of starvation of 39 hours each.

[1] This subject on several other occasions has given blood-sugar curves completely normal in every respect.

Exp. 6. *Metabolism of 50 g. glucose by Subject II after 39 hours' starvation* (Table VIII).

Table VIII.

Blood analysis.

Time after glucose mins.	Blood-sugar mg. per 100 cc. Capillary	Blood-sugar mg. per 100 cc. Venous	Plasma-CO_2 combining power vols. %	Plasma-inorganic-P mg. per 100 cc.	Lactic acid mg. per 100 cc. whole blood
0	95	83 (2)	73·9	3·32	13·1
30	120	130 (3)	72·9	3·20	12·6
60	152	152 (5)	72·0	2·99	14·4
90	154	—	—	—	—
120	178	128 (10)	65·3	2·86	13·5
158	130	—	—	—	—
190	81	—	—	—	—

Venous blood taken ( ) minutes after capillary blood.

Respiratory metabolism.

Time after glucose mins.	Urinary nitrogen mg. per min.	Non-protein R.Q.	O_2 cc. per min.	g. metabolised per hour: Protein	Fat	Carbohydrate
Basal	13·04	0·71	252	4·89	5·30	1·70
Basal	11·25	0·76	248	4·22	4·59	2·34
25	11·09	0·73	268	4·16	5·69	1·15
55		0·77	270	4·16	4·95	3·12
88	15·00	0·81	254	5·63	3·38	4·19
118	12·12	0·81	270	4·55	4·00	4·96
140	11·35	0·86	263	4·26	2·84	7·58

Urinary findings.

Time after glucose mins.	Titratable acidity in cc. $N/10$ per hour	Rothera's reaction
0	−4·5 (alk.)	Positive
36	−1·3 (alk.)	Strong
70	14·1	Trace
101	9·7	Negative
135	5·3	,,
190	2·7	,,

Attention may be drawn in this experiment to the facts that capillary-venous blood-sugar differences did not become established until well over an hour from the taking of the glucose, that the alkali reserve was high to begin with and at no time showed evidence of an acidosis, and further that the plasma-inorganic phosphorus fell steadily for 2 hours. The blood-lactic acid, as in the previous experiment, showed no change of note. It is also to be noted that the subject at the time of ingestion of the glucose was excreting an alkaline urine (morning "alkaline tide"). The cause of this phenomenon has been sought in the early morning hyperpnoea with resultant loss of CO_2 and hence the compensating loss of alkali, which sometimes occurs. It has also been attributed to secretion of gastric HCl on rising after sleep. The figures in Table VIII rather suggest that the morning alkaline tide, when it does occur, is associated with a high alkali reserve, which may be due to a temporary mobilisation of base, to balance a CO_2 retention during sleep. The fall in alkali reserve is at once reflected in a marked excretion of acid in the urine.

The ketonuria was not very marked and disappeared completely in about 70 minutes after the glucose was taken. In spite of these facts the respiratory findings show a persistently low oxidation response to carbohydrate.

Since the R.Q. was 0·86 at the end of this experiment it was necessary to repeat the experiment and continue until it was clear that no further rise in R.Q. was going to take place.

Exp. 7. *Metabolism of 50 g. glucose by Subject II after 39½ hours' starvation* (Table IX).

Table IX.

Time after glucose mins.	Capillary blood-sugar mg. per 100 cc.	Non-protein R.Q.	O_2 cc. per min.	g. metabolised per hour		
				Protein	Fat	Carbohydrate
Basal	85	0·77	255	5·35	4·37	2·63
30	115	0·74	258	4·91	4·90	2·32
68	146	0·76	262	4·91	4·66	2·38
103	166	0·856	245	5·00	2·62	6·09
142	100	0·854	274	5·00	3·00	6·96
180	78	0·79	266	3·47	4·77	4·03
225	67	0·695	259	3·72	6·05	0

The initial ketosis in this experiment was very slight and rapidly disappeared after the glucose was taken. The blood-sugar curve was somewhat prolonged but not markedly abnormal. The fact that some 3¾ hours after the glucose the blood-sugar was 67 mg. per 100 cc. shows that the mechanism for removal of sugar from the blood was now extremely active. The respiratory data are, however, unequivocal. The maximum rise in R.Q. was to 0·86 in 103 minutes, after which it fell to fat levels and showed no sign of rising again. Comparison of Tables VIII and IX with Table VII leaves no doubt that, on the usual assumptions as to the significance of the R.Q., there was a great depression of the power to deal immediately with carbohydrate.

The most obvious assumption in explanation of these facts is that in conditions of starvation there is a deficiency of insulin. If we take the view that insulin inhibits gluconeogenesis, then the fact that in starvation this process becomes more active would strengthen the belief that some inhibition of insulin secretion or activity occurs. It is known that insulin produces a marked rise in R.Q. in the normal subject starved for 16 hours [Goldblatt and Ellis, 1931] so that we should expect a rapid readjustment to normal reactions to glucose administration if both insulin injection and glucose ingestion were practised on the subject starved as in the previous experiment. The results of such an experiment are given in Table X.

Exp. 8. Subject II was starved for 39 hours. Basal determinations having been made, 10 units of insulin were injected subcutaneously; 15 minutes later 50 g. glucose were taken and the observations were made.

In this experiment there was considerable ketonuria at the beginning; this ceased completely about 1½ hours, but began to reappear some 4 hours after the glucose was taken. Glycosuria was absent throughout.

Table X.

Time after glucose mins.	Non-protein R.Q.	O_2 cc. per min.	g. metabolised per hour		
			Protein	Fat	Carbohydrate
Basal	0·85	249	4·84	2·80	6·10
25	0·75	263	4·73	4·93	1·96
59	0·78	263	6·02	4·40	3·35
91	0·78	252	6·02	3·71	2·83
130	0·86	251	4·77	2·66	6·61
170	0·87	237	3·09	2·62	7·49
206	0·84	246	3·09	3·65	6·31
242	0·76	258	2·11	5·26	3·59

Time after glucose (mins.)	0	35	70	100	150	195	
Blood-sugar	81	109	143	139	109	51	(Hypoglycaemic symptoms with spontaneous recovery)

The peculiar fact emerges from this experiment that, whilst insulin completely prevented any manifestation of intolerance to sugar as far as the blood-sugar is concerned, it did not simultaneously readjust the oxidative processes.

Table XI gives the maximum rises in O_2 utilisation and maximum rises in heat production in the above experiments after 50 g. glucose.

Table XI.

	Maximum rise in O_2 cc. per min.	Maximum rise in heat production cals. per hour	Maximum R.Q. (non-protein)
Control period	26·9	9·7	1·04
39 hours' starvation...	18·4	6·3	0·85
39½ hours' starvation	19·0	6·5	0·85
39 hours' starvation and 10 units insulin...	14·0	3·1	0·87

Percentage increase in heat production after giving glucose.

Control. Basal 73·5 Cals.		39 hours' starvation. Basal 71·3 Cals.		39½ hours' starvation. Basal 73·2 Cals.		39 hours' starvation. Insulin 10 units. Basal 72·6 Cals.	
Time mins.	% increase	Time mins.	% increase	Time mins.	% increase	Time mins.	% increase
28	4·6	25	7·0	30	0	25	3·2
58	0	55	8·7	68	2·3	59	4·3
93	14·7	88	2·8	103	0	91	0
130	10·9	118	9·2	142	8·9	130	0
—	—	140	7·4	180	4·2	170	−4·3
—	—	—	—	225	0	206	−1·8
—	—	—	—	—	—	242	1·6

In interpreting Table XI it is important to realise how steady the basal metabolism and oxygen consumption of this subject were at the beginning of each experiment. Of six determinations the mean percentage deviation from the mean (72·6) was 1·2 (max. 2·7–min. 0). There can thus be no reasonable doubt that some diminution in the so-called specific dynamic action of glucose becomes manifest in starvation. It is further evident that the injection of insulin does not immediately readjust the mechanism otherwise than to bring down the blood-sugar. This is the more strange since, as we found

in previous work [1931], insulin alone can, in a normal subject starved for 16 hours, raise the R.Q. from a basal non-protein value of 0·80 to 1·01 with a simultaneous increase in heat production of about 12 % in about an hour.

It is further of interest to note that whilst with glucose alone after starvation the calorie increase reached about 9 % above basal values, with insulin and glucose it attained only 4 % and that for a relatively short time.

The marked lowering of blood-sugar after insulin associated with a failure of the rate of oxidation of carbohydrate to rise to a greater extent than without insulin is extremely difficult to explain on the usually accepted theories of action of this hormone. We must seek the cause in the tissues.

If, as we believe, the diminished tolerance for carbohydrate observed in starvation is not explicable on the basis of an acidosis, then it is not admissible to suppose that the failure of insulin to readjust carbohydrate oxidation in these conditions is due to changes in tissue reaction. Dann and Chambers [1930] found that, after prolonged periods of starvation, even 60 units of insulin in a 10 kg. dog did not bring the oxidation of carbohydrate (50 g. glucose) to normal. Thus, whereas the normal dog after receiving this amount of sugar could metabolise about 4·7 g. glucose per hour, the starved animal oxidised only 0·6 g. glucose per hour, and when starved and given 60 units insulin with the sugar it oxidised only 2·1 g. carbohydrate per hour. There was obviously a marked improvement in the power to metabolise sugar but the value obtained fell far short of the normal. They also found that after about 3 weeks' starvation the rate of oxidation of carbohydrate remained deficient even during 3 days with daily doses of 50 g. glucose, whereas insulin simultaneously injected readjusted peripheral oxidation completely on the second day. Thus the failure of insulin to produce its typical effects was limited to the first day of the experimental period after starvation (19 days). In our experiment the preliminary period of starvation was just sufficiently long to make manifest the deficient power to oxidise glucose, and there was already a marked failure in oxidation response to insulin, in spite of the fact that the blood-sugar fell to hypoglycaemic levels. Since there is no reason to suppose that the absorption of glucose from the gut is deficient (the contrary is more probable) after starvation, we must suppose either that some essential stage in the metabolism of sugar is delayed or that the tissues after starvation differ fundamentally in some respect from normal tissues. The results of Eisner (*vide supra*) seem to us to offer strong evidence in favour of the former view, that is to say that there is a delay in the formation of that concentration of glycogen which permits of what is called normal metabolism. If we regard insulin as the physiological stimulus to the reactions which lead to both glycogen formation and carbohydrate oxidation, then it appears probable that in the conditions of these experiments the predominant effect of insulin is to promote the former of these results. Later, when adequate concentrations of glycogen are formed, the oxidative response to sugar becomes normal; a condition of things which may result, as we have seen, some

time only after the administration of sugar. The fact that even during starvation intolerance very considerable differences in capillary and venous blood-sugar are demonstrable even when oxidation of carbohydrate is inadequate, indicates that retention of sugar in the tissues must be actively proceeding. It is known that in animals the injection of insulin during carbohydrate absorption leads always to a smaller deposition of glycogen in the liver than when the carbohydrate is given alone. This fact is interpreted as due to the increased deposition and oxidation of carbohydrate peripherally. If this can be assumed to apply to the human organism then the results in Table X must indicate increased deposition of carbohydrate in the tissues, probably first as free sugar and later as glycogen, the oxidative response occurring considerably later.

Summary and conclusions.

1. Evidence is presented to show that the intolerance for carbohydrate manifested after periods of about 40 hours' starvation is not due to either ketosis or acidosis.

2. The observation of Dann and Chambers that insulin does not completely re-establish normal carbohydrate oxidation in dogs after prolonged starvation, is shown to have its counterpart in human subjects even after only 40 hours' starvation.

3. In explanation of these facts it is considered unnecessary to assume any special changes in the tissues other than those incident to a fall in glycogen content. It is suggested that in the starving organism there is a temporary dissociation of the two mechanisms initiated by insulin, *viz.* the formation of peripheral glycogen and the oxidation of carbohydrate, the second effect being manifest only when the first has attained adequate proportions.

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CXIX. THE DETERMINATION OF MILK-PROTEINS BY FORMALDEHYDE TITRATION.

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The formaldehyde method for the determination of milk-proteins, originally proposed by Steineggar [1905], has been much employed since its inception on account of its ease, rapidity, and reasonable accuracy. In the original method, sodium hydroxide appears to have been the alkali employed in the titration. Richmond [1906, 1911] later recommended strontium hydroxide as giving a sharper end-point, and remarked further that the titrations obtained with this substance were considerably higher—on the average by about 10 %—than those obtained with sodium hydroxide. This difference he attributed primarily to the differing degrees of hydrolysis of the strontium and sodium salts of the milk-proteins and of the products formed from them by the action of formaldehyde, and, to a slight extent, to similar differences in the degrees of dissociation of the strontium and sodium salts of the acids usually present in commercial formaldehyde. It appears however that there is an additional and probably more important factor which plays a part in the titration differences mentioned, namely, the formation of tricalcium phosphate during the formaldehyde titration. It has long been recognised that this precipitation occurs in the determination of milk-acidity in the usual way, and that the extent to which it occurs, and therefore the resulting titratable acidity of the milk, depends greatly on the conditions of titration. Thus the diminished acidity resulting from the dilution of milk has been ascribed by Sommer and Menos [1931] to the greater solubility, *i.e.* diminished precipitation, of tricalcium phosphate in the diluted medium. Similarly the very different milk-acidities found by Roeder and Radoi [1925] according to whether sodium or calcium hydroxide had been used for the neutralisation have been attributed to the different degrees of phosphate precipitation in the two cases, the higher acidities occurring, as was to be expected, when the alkaline earth was employed, and conditions were more favourable to increased precipitation.

It is clear that such an influence, evident enough in the neutralisation of milk, must also make itself felt in the subsequent formaldehyde titration. The latter will therefore give a composite result, dependent mainly on the protein content of the milk but also influenced by the presence of phosphates

to an extent which will vary with the individual milk sample and the type of alkali used for the titration.

Confirmation of this view is furnished by determinations of the formaldehyde titration of proteins in the presence and absence of phosphates, with various alkalis (Table I) and also by determinations on some samples of milk to which phosphate had been added.

Table I.

Solution	Formaldehyde value cc. N alkali per 100 cc. sol.	Alkali
Egg-albumin A	1·37	$Sr(OH)_2$
,, +10 % $M/5$ alkali phosphate (p_H 6·6)	1·60	,,
Egg-albumin B	1·80	,,
,, +20 % serum*	2·00	,,
Egg-albumin C	1·20	NaOH
,, +20 % serum*	1·27	,,
Calcium caseinogenate	1·60	$Sr(OH)_2$
,, +10 % serum*	1·71	,,
Milk A	1·88	,,
,, +10 % serum*	1·98	,,
Milk B	1·71	NaOH
,, +10 % serum*	1·77	,,

* Prepared by adding to the protein solution in order equal volumes of (*a*) 0·3 M $CaCl_2$ and (*b*) a solution containing 0·25 M sodium citrate, 0·15 M KH_2PO_4, and 0·15 M Na_2HPO_4. The mixture diluted ten-fold corresponds approximately in salt content to that of average milk-serum.

It is evident from these results that the use of $Sr(OH)_2$ for the determination of formaldehyde values of proteins in the presence of phosphate, *e.g.* in milk, is open to objection, and it would also appear that this applies, though to a less extent, to alkali hydroxides. It would seem advisable therefore that milk before determination of its formaldehyde values should be treated so as to obviate the disturbing effects of phosphate. For this purpose, the addition of neutral potassium oxalate (2 % of a saturated aqueous solution), recommended by Van Slyke and Bosworth [1914] for similar reasons for the determination of milk-acidity, suggested itself, and its influence on the formaldehyde titration has therefore been examined.

In Table II are shown some formaldehyde values of milk determined by all three methods, *i.e.* by NaOH, with and without the addition of oxalate, and by $Sr(OH)_2$.

Table II.

Material	Formaldehyde value cc. N alkali per 100 cc. milk		Alkali
	A	B	
Milk alone	1·69	1·71	NaOH
,, +2 % sat. oxalate	1·80	1·92	,,
Milk alone	1·94	2·01	$Sr(OH)_2$

In these experiments the use of oxalate is seen to give values intermediate between those obtained with sodium and strontium hydroxides, but closer

perhaps to those of the latter. This result was somewhat unexpected, as it was thought likely that the oxalate values would be the lowest of the three, on account of the exclusion of any phosphate precipitation. The most obvious explanation of the values actually obtained seemed to be connected with the hydrolysis of the protein and formaldehyde-protein salts to which Richmond [1906, 1911] drew attention in this connection, the degree of hydrolysis being perhaps largely influenced by the very much increased alkali ion concentration arising from the introduction of the oxalate.

To investigate the effect of such variations in the extent of hydrolysis, experiments were next carried out with pure proteins—egg-albumin and calcium caseinogenate—under similar conditions.

Typical results are shown in Table III.

Table III.

Solution	Formaldehyde value cc. *N* alkali per 100 cc. sol. A	B	C	D	Alkali
Egg-albumin	1·93	1·37	—	—	$Sr(OH)_2$
,,	1·83	1·31	2·00	—	NaOH
,, +4 % sat. oxalate	—	—	2·04	—	,,
Calcium caseinogenate	2·00	2·03	1·22	1·42	$Sr(OH)_2$
,, ,,	1·90	1·96	1·20	1·38	NaOH
,, ,, +4 % sat. oxalate	2·00	2·00	1·22	1·43	,,

From these it is seen that, in the absence of phosphates, the use of strontium hydroxide leads to somewhat higher formaldehyde values (about 3–5 %) than are obtained with sodium hydroxide, but that if much oxalate is present the values obtained with NaOH increase, and approximate to those of $Sr(OH)_2$. The values obtained with the two hydroxides do not differ, however, very greatly, and it is evident therefore that variations in hydrolysis resulting from their use cannot account for more than 1/3 to 1/2 of the total difference usually observed in the formaldehyde titration of milk.

The residual and greater part of the titration difference appears to arise from the presence of phosphate, as suggested earlier.

It may be remarked here that the precipitation of tribasic phosphate during titration obviously cannot account for the formaldehyde titration of oxalated milk (using NaOH) being so much higher than that of untreated milk—approximating in fact to that obtained with $Sr(OH)_2$. The explanation of this point will be considered later.

As it seemed of interest to determine to what extent each of the protein salts of milk contributed to the hydrolysis discussed above, some further experiments of a qualitative nature were made with egg-albumin and caseinogen. Solutions of these proteins alone and solutions containing formaldehyde were neutralised to phenolphthalein with strontium hydroxide, and gradually increasing amounts of neutral potassium oxalate were added to each. The indicator tint in the neutralised protein solution was scarcely affected by this

treatment, but that in the solution containing formaldehyde deepened considerably on the first small addition of oxalate (*i.e.* on conversion of the strontium into a potassium salt) and showed a marked tendency to lighten again as the amount of oxalate added was increased. The difference in the formaldehyde titration values with NaOH and $Sr(OH)_2$ in the absence of phosphates appears therefore to be mainly due to the different degrees of hydrolysis of their respective formaldehyde-protein salts.

The experiments on hydrolysis make it clear that if oxalate is to be used, the amount employed must be definitely specified. Van Slyke and Bosworth [1914] prescribed an addition of 2 % of saturated potassium oxalate for the determination of the titratable acidity of milk, and for the determination of formaldehyde values this amount seemed adequate with most samples. An occasional sample was met with, however, in which the addition of more than this proportion of oxalate was required to develop a minimum titratable acidity—and therefore a maximum removal of calcium—and a somewhat higher proportion of saturated oxalate, *viz.* 4 %, was therefore decided on.

This amount has proved adequate for all milks met with up to the present, and has the further advantage that the formaldehyde values obtained by its use agree fairly closely with those obtained by Richmond's $Sr(OH)_2$ titration.

Table IV gives the results of a number of comparative estimations of the formaldehyde value of milks, carried out with and without the addition of 4 % saturated potassium oxalate, the alkali throughout being sodium hydroxide.

Table IV.

Milk No.	Formaldehyde value cc. *N* alkali per 100 cc. milk: With oxalate	Without oxalate	Ratio	Milk No.	Formaldehyde value cc. *N* alkali per 100 cc. milk: With oxalate	Without oxalate	Ratio
1	1·82	1·68	1·09	16	2·26	2·04	1·11
2	2·00	1·84	1·09	17	1·81	1·63	1·11
3	1·84	1·69	1·09	18	1·94	1·75	1·11
4	1·74	1·60	1·09	19	2·02	1·82	1·11
5	1·96	1·80	1·09	20	2·61	2·35	1·11
6	2·20	2·01	1·10	21	2·25	2·02	1·11
7	1·95	1·78	1·10	22	1·93	1·73	1·12
8	2·03	1·85	1·10	23	2·38	2·13	1·12
9	1·91	1·74	1·10	24	1·90	1·70	1·12
10	2·01	1·83	1·10	25	1·84	1·64	1·12
11	2·00	1·82	1·10	26	2·13	1·90	1·12
12	2·07	1·88	1·10	27	2·30	2·05	1·12
13	1·94	1·76	1·10	28	1·92	1·71	1·12
14	2·02	1·83	1·10	29	2·36	2·09	1·13
15	1·97	1·77	1·11	30	1·98	1·76	1·13
						Mean ratio	1·107

The titration values of the oxalated milks are seen to be considerably (9–13 %) the higher—a somewhat unexpected result. From the earlier experiments on pure protein solutions it appeared that (*a*) the formaldehyde values obtained with NaOH alone were only slightly (3–5 %) lower than those obtained in the presence of oxalate (see Table III), and that (*b*) the

addition of phosphate was capable of increasing slightly the titrations with NaOH alone (Table I). It was thought therefore that these two factors, acting in opposition when milk was under investigation, should result in the formaldehyde values being approximately the same whether oxalate were present or not. The results obtained in Table IV, showing on the contrary the oxalate titration to be about 11 % the higher, suggested that there were other and important factors affecting the formaldehyde titration of milk which had escaped consideration.

The only milk constituent besides protein and soluble phosphate which appeared in any way likely to play such a part was colloidal calcium phosphate, which from its close connection with the calcium caseinogenate of milk might be conceived to influence in some way the interaction of formaldehyde with the protein, or the degree of titratable acidity developed therefrom. An attempt was therefore made to examine its effect on the formaldehyde value of caseinogen, and for this purpose a caseinogen-calcium phosphate complex was prepared on the lines indicated by Porcher [1930]. The method used consisted briefly in dissolving caseinogen (Hammarsten) in an excess of lime water (36 mg. CaO per 1 g. caseinogen) and running $M/20$ phosphoric acid slowly and with vigorous stirring into this alkaline solution until a p_H value of slightly less than 7·0 was attained. To obviate as far as possible partial decomposition of the caseinogen by the alkali during the preparation of the complex, the protein was dissolved originally in about one-half the total quantity of lime water needed for the complex, the solution cooled in ice, and the remainder of the lime water, similarly cooled, added almost immediately before proceeding to run in the phosphoric acid. During this stage the mixing vessel was also cooled with ice.

Table V shows the formaldehyde values determined in the various ways already employed, of a series of caseinogen-calcium phosphate complexes

Table V.

Solution	Protein %	Formaldehyde value cc. N alkali per 100 cc. sol.	Alkali used	Ratio $\frac{\text{Protein}}{\text{Formald. value}}$
Caseinogen-calcium phosphate complex A	—	1·20	NaOH	—
	—	1·42	Oxalate and NaOH	—
	—	1·45	$Sr(OH)_2$	—
Caseinogen-calcium phosphate complex B	2·24	1·10	NaOH	2·04
	2·24	1·27	Oxalate and NaOH	1·76
	2·24	1·26	$Sr(OH)_2$	1·78
Caseinogen-calcium phosphate complex C	2·44	1·20	NaOH	2·03
	2·44	1·38	Oxalate and NaOH	1·77
	2·44	1·40	$Sr(OH)_2$	1·76
Caseinogen-calcium oxalate complex C	2·32	1·30	NaOH	1·78
	2·32	1·32	Oxalate and NaOH	1·76

prepared in this fashion, and, for comparison, those of a similar complex in which the calcium phosphate is replaced by calcium oxalate (by neutralising

the excess calcium hydroxide with dilute oxalic acid instead of $M/20$ phosphoric acid). Some protein determinations made by the Kjeldahl method are included and assist the interpretation of the results.

The results with the caseinogen-calcium phosphate complex are seen to be somewhat similar to those obtained with milk and make it evident that the presence of colloidal calcium phosphate can depress the formaldehyde titration of caseinogen with NaOH to a value much below that found for caseinogen in its absence. The titrations obtained on addition of potassium oxalate are practically identical whether colloidal phosphates were originally present or not, as is to be expected. Those obtained with $Sr(OH)_2$ as alkali seem also to be relatively independent of the presence of colloidal phosphates, but the observations in this case must be interpreted to mean that the reduction in the formaldehyde titration through the presence of colloidal phosphates and the influence of these on caseinogen is approximately counterbalanced by the additional alkaline earth used in precipitating some of the soluble, and also some of the colloidal, dicalcium hydrogen phosphate as tricalcium phosphate.

The results obtained with sodium hydroxide, which show that the acidity developed on adding formaldehyde to a solution of calcium caseinogenate is markedly reduced in the presence of colloidal calcium phosphate, appear to favour the view that the caseinogen and colloidal phosphate in the complex and also in milk are not present as isolated substances, but exist in some kind of chemical union, the exact nature of which is not yet clear. That their relation however is something more specific than one of mere colloidal protection of the phosphate by the protein seems to be inferable from the entirely different behaviour of the caseinogen complex containing protected calcium oxalate instead of phosphate, and from further experiments made on colloidal calcium phosphate protected by other proteins. Thus, a 5 % gelatin solution containing colloidal phosphate, whether prepared by the interaction of disodium hydrogen phosphate and calcium chloride, or by that of calcium hydroxide and phosphoric acid, gives practically identical formaldehyde values with NaOH, with or without addition of potassium oxalate.

The general practical conclusion to be drawn from these results is that the formaldehyde value of milk, as hitherto estimated, is a composite value, determined largely by the protein content of the milk, but affected in various directions by both colloidal and soluble phosphates—to an extent which depends on the type of alkali used for the titration. It seems advisable therefor in using this value for the determination of protein in milk, to eliminate these disturbing features as far as possible, a result easily attained by the use of oxalate. Not only is the correlation between protein and formaldehyde value greatly improved by this means (see Table VI), but the titration is also facilitated by the improved sharpness of the end-point and the practical elimination of that fading (due to slow formation of tribasic phosphate) which otherwise accompanies the use of NaOH and more particularly $Sr(OH)_2$.

In Table VI will be found the results of a number of protein determinations (N × 6·38), and formaldehyde values of mixed milks, and in Table VII the results in abridged form of a somewhat similar but more extensive series of estimations made on the milks of individual cows (Shorthorn breed).

Table VI.

Milk mixed	Protein per 100 cc. milk	Formaldehyde value cc. *N* alkali per 100 cc. milk		Ratio $\frac{\text{Protein}}{\text{Formald. value}}$	
		With oxalate	Without oxalate	With oxalate	Without oxalate
1	3·44	2·00	1·82	1·72	1·89
2	3·40	1·97	1·76	1·73	1·93
3	3·43	1·98	1·76	1·73	1·95
4	3·36	1·94	1·76	1·73	1·91
5	3·19	1·84	1·64	1·73	1·94
6	3·14	1·81	1·63	1·74	1·93
7	3·41	1·95	1·78	1·75	1·92
8	3·33	1·90	1·70	1·75	1·96
9	3·05	1·75	1·60	1·75	1·90
10	3·53	2·01	1·83	1·76	1·93
			Mean	1·74	1·926

Table VII.

Ratio $\frac{\text{Protein}}{\text{Formald. value}}$ (using oxalate)	No. of samples (milk of single cows)	Ratio $\frac{\text{Protein}}{\text{Formald. value}}$ (using oxalate)	No. of samples (milk of single cows)
1·63	1	1·75	6
1·64	1	1·76	3
1·65	1	1·77	3
1·67	1	1·78	3
1·69	3	1·79	1
1·70	5	1·80	2
1·71	2	1·82	1
1·72	3	1·84	1
1·73	3	1·87	1
1·74	5	Mean 1·74	46

The formaldehyde titrations (using oxalate) were made with *N*/10 sodium hydroxide on 10 cc. samples of milk to which 0·4 cc. saturated aqueous potassium oxalate, and 0·5 cc. of 0·5 % phenolphthalein as indicator, were added. After standing for about 2 minutes the milk was neutralised, the end-point being a faint pink which was matched with that of a similar quantity of milk and oxalate tinted by addition of a drop of dilute alcoholic magenta[1]. 2 cc. 40 % formaldehyde were then run in and the titration with NaOH continued to the same tint as before. The acidity of the formaldehyde must of course be subtracted from the milk titration and may be determined by similarly titrating 2 cc. formaldehyde in 10 cc. water with *N*/20 alkali. The recorded values in Tables VI and VII are in all cases the mean of two or more estimations. Where oxalate was used the agreement of duplicates was almost exact—the difference in no case amounting to as much as 0·05 cc.

[1] The depth of tint given by one drop of this magenta solution to 10 cc. water was approximately the same as that developed in 10 cc. of a borate buffer (p_H 7·9) containing 0·5 cc. of 0·5 % phenolphthalein.

The results with mixed milks (Table VI) and with individual milks (Table VII) agree very well, giving an almost identical ratio $\frac{\text{protein}}{\text{formald. value}}$ of approximately 1·74 where oxalate is employed. The average value obtained with NaOH alone (without oxalate) for mixed milks may be expressed as 1 cc. *N* NaOH = 1·926 g. milk-protein, and agrees very well with that found by Steineggar for NaOH, quoted by Richmond [1906], namely 1 cc. *N* NaOH = 1·933 g. milk-protein. It will be noted from Table VI that the ratios $\frac{\text{protein}}{\text{formald. value}}$ obtained by the use of oxalate show much less variation from sample to sample than those for NaOH alone. This is in line with the somewhat considerable degree of variation in the ratios of the values obtained with these two types of titration (see Table IV) and is, of course, to be expected from what has been seen of the various factors which influence the formaldehyde titration in the absence of oxalate.

The protein values of 1 cc. *N* alkali, using oxalate, obtained with the milks of individual cows vary more, naturally, than those from mixed milks, as may be seen by comparing the data of Tables VI and VII. Some of the individual samples, which deviated most from the mean, were from cows approaching the end of lactation, and in these the percentage of protein was unusually high, and presumably the ratio of caseinogen to albumin also abnormal. As the acidities developed by equal weights of albumin and caseinogen on addition of formaldehyde are very different [*cf.* Richmond, 1906], marked variation in their relative amounts in milk is bound to cause a displacement of the protein/formald. value ratio, which cannot be obviated by the use of oxalate. The formaldehyde method in either its original form or the modified one suggested here is thus suited best to average normal or mixed milk. The question of further modification to cover abnormal samples is however receiving attention.

The formaldehyde and protein values given in the foregoing tables have all been expressed in terms of the same volume. For practical use the factor 1·74 requires to be modified slightly to express the protein as a percentage by weight. The standard 10 cc. pipette used to measure the milk for both formaldehyde and Kjeldahl determinations was found to deliver 10·25 g. of average milk (sp. gr. 1·032). The factor for converting the observed formaldehyde value (expressed as cc. *N*/10 alkali per quantity of milk delivered by a 10 cc. pipette) into percentage protein by weight therefore becomes $\frac{1{\cdot}740}{1{\cdot}025}$ or 1·70 for milk of average density.

Summary.

1. The formaldehyde titration for the determination of milk-proteins, as usually carried out, has been shown to be considerably affected in different directions by the presence in milk of (*a*) soluble or easily reacting phosphates, and (*b*) colloidal phosphates. The former bring about an increase in the

formaldehyde value, particularly if an alkaline earth hydroxide is employed for the titration, and the latter a decrease.

2. The effect of the colloidal phosphate on the formaldehyde titration seems to favour the view that the calcium caseinogenate and colloidal phosphate of milk exist in some form of chemical combination.

3. The disturbing effects of both classes of phosphate can be eliminated by treating milk with an oxalate, prior to carrying out the formaldehyde titration. A suitable procedure is suggested for the purpose.

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CXX. THE COMPOSITION OF THE TISSUES OF ALBINO RATS TREATED WITH ALKALINE ANTERIOR PITUITARY EXTRACTS.

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As Evans and Long have shown [1921], a bovine anterior pituitary extract, prepared and administered in the proper manner, induces a pronounced increase of weight in rats. This was confirmed by Hewitt [1929], Johnson and Everett [1929], and Gordon and Handelsmann [1930]. The phenomenon has been reproduced in dogs by Putnam and Benedict [1928], Reichert [1929], Cushing and Teel [1929], and in pigeons by Riddle [1931]. The negative results of experiments which have been published by some authors [Larson *et al.* 1929, Korenchevsky 1930] may be attributed either to unsuitable preparation of extracts (addition of antiseptics, heating, *etc.*) or to the incorrect choice of experimental objects. (With regard to rats, for instance, the growth curve must have reached a plateau in order that the phenomenon may be demonstrated at all.) According to Evans and Long [1922], the induced increase in weight is due partly to the formation of fat, partly to the growth of organs and bone. The majority of later writers have agreed in finding an increase in the weight of certain parenchymatous organs and in the dimensions of the skeleton, changes which have been interpreted as acromegaly by some investigators. There are no analyses, however, of the composition of the tissue of animals treated with such extracts, and the organ weights and bone measurements published cannot be statistically treated, so for the present it must suffice to state that potent pituitary extracts induce a statistically significant increase of weight in suitable experimental objects. On the other hand the past few years have seen the appearance of a number of reports indicating that this weight increase is not permanently established. Hewitt [1929] was, we believe, the first to make the observation that discontinuation of the injections brings about an immediate and rapid falling off in weight. In the same year Downs and Geiling [1929] announced that experimental giant mice deprived of water fall so much in weight in the course of 24 to 48 hours that they approach the controls, and Lee, Teel and Gagnon [1929] similarly maintain that experimental giant rats lose much more in weight during the first 22 hours after death than the controls. These observations seem to indicate that the increase of weight is exclusively or principally due to the retention of water, which is

excreted as soon as the injections are stopped, and they have resulted in doubt whether any growth hormone at all occurs in the extracts employed.

In our laboratory we have long been familiar with the fact that discontinuation of extract injections involves immediate and marked loss of weight (Fig. 1). In our experience (confirmed quite recently by Evans and Simpson [1931]) this loss, however, amounts to only approximately 35 % of the weight increase that has been obtained. If this is true, it is extremely improbable that the induced gigantism should be due to water retention alone. Since there are no known investigations into the composition of the tissues of anterior pituitary-treated animals, we report below on the result of a series of dry-matter and total-N determinations on albino rats treated with extract, with parallel controls.

Details and technique of experiments.

The material used was 8 albino rats for the tests (6 ♂ and 2 ♀) and 7 albino rats for control (6 ♂ and 1 ♀). Unfortunately, at the time it was not possible to secure couples of the same litter. The significance of this will be referred to later. When the experiments were started (3/2/31), the average weight of the experimental animals was 75 g. and of the controls 78 g. The food was the standard diet of Gudjonsson [1930], consisting of powdered skim milk 30 %, ground rice 40 %, yeast 15 %, and cocoa butter with liver oil 15 % (liver oil 0·3 %). Food and water were given *ad lib.* and the quantity of water consumed was measured daily.

The extract employed was prepared according to Evans with certain modifications. The pituitary glands were removed as quickly as possible after the slaughtering of the cows (less than half an hour after killing) and put on ice at once. Experience had shown that this latter point is of vital importance to the obtaining of potent extracts. The Seitz-filtered extract is a slightly yellowish, sometimes reddish, somewhat cloudy fluid of p_H about 6·7 and containing about 1·4 % dry-matter (N 0·2 %). It produces colour changes in axolotls and frogs and thus undoubtedly contains posterior lobe (*intermedia*) hormones. Titration of the melanophore-expanding principle on *Rana temporaria* showed that 1 cc. of extract corresponded to 0·13 cc. of pituitrin, 0·13 cc. pitressin and 6·6 cc. of pitocin (Parke Davis).

Dose: For the first three months each rat was given 0·5 cc. of extract daily, injected intraperitoneally, corresponding to about 0·1 g. of fresh anterior lobe. Later on 1 cc. was given daily.

The rats were killed (with coal-gas) over the period 13/10–31/10. One rat was killed every day, experimental animal and control animal being killed at intervals of 24 hours. Immediately after death the animal was weighed, then placed in the ice-box for 24 hours—which very much simplifies the subsequent mincing. After 24 hours the animal was weighed again. The average loss of weight during chilling was 0·5 g. (max. 3 g.), an error which of course can easily be eliminated by using a correction in every case. The mincing process consisted in grinding the whole rat (stomach, guts and skin) a sufficient number of times (average 10) in an ordinary sausage machine. As a rule it was possible to complete the mincing without the least loss of dry matter.

If we call the weight prior to mincing b, that of the instruments used (mincing machine, spatula, forceps, scissors, *etc.*) c, and that of minced meat + instruments after mincing d, the loss of weight through evaporation during mincing is $b + c - d$, and the weight of the meat $d - c$. The loss by evaporation was on an average 3 to 5 g. (max. 15 g.). This error, of course, can also be eliminated by correction.

The mince thus obtained was an extremely homogeneous, red mass, although some fragments of hair were distinguishable.

The following samples were taken of this mixture as quickly as possible: (1) 4 samples of about 0·5 g. for N analyses, (2) 2 of 1 g. for dry matter, (3) 4 (reserve samples) of 1 g. All samples were weighed to an accuracy of 0·1 mg.[1], and of course the samples were taken from various parts of the mince.

[1] In reality such extreme accuracy is superfluous for the present purpose.

The N determinations were made according to Kjeldahl's method. The samples were weighed on dried, cleaned fragments of object-glass, which were immediately thrown into the incineration-tube so that waste of mince through transfer was entirely avoided.

The real N % (X) was calculated from the N % found (f) by means of the following correction:

$$X = \frac{f.e}{b} \cdot \frac{b}{a} = \frac{f.e}{a}$$

where a and b are the weight of the rat before and after chilling, e the weight of the total mince (e/b correction for evaporation when mincing and b/a correction for evaporation when chilling).

The dry matter tests were performed in weighing-tubes. All samples were dried for 4 days at about 90°. When this was completed, small irregular fluctuations were observed of $< 0{\cdot}001$ g. With the quantities employed (1 g.) these involve an error of $< 0{\cdot}1$ %, which may be regarded as insignificant for present purposes.

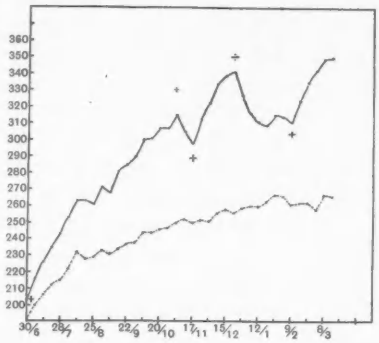

Fig. 1.

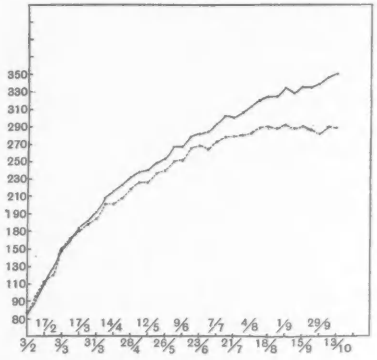

Fig. 2.

Fig. 1.

•—•—•—• Weight curve of 10 Wistar rats (4 ♂ and 6 ♀) injected with anterior pituitary extract for 7 months.

•– – •– – •– – • Controls. 10 Wistar rats (4 ♂ and 6 ♀).

÷ means injections stopped. + means injections started.

Fig. 2. Weight curves of rats used for the analyses.

•—•—•—• Injected rats. •– – •– – •– – • Controls.

Results.

The average weights of experimental animals and controls are given graphically on Fig. 2. It will be seen that the curves almost completely coincide in the first two months, which argues that the spontaneous growth energy has been the same for both treated and untreated animals, and makes it improbable that the subsequent dissociation of the curves is caused by accidents. In this connection we may refer to Fig. 1, which illustrates the effect of the extract upon a series of Wistar rats consisting of couples of the same litter. On the whole, extracts prepared in the manner described have in our laboratory without exception given a distinct difference in the average weight of experimental and control animals in favour of the former. It will be noticed that the curves do not deviate until at about 200 g., which agrees with the experience

of other laboratories. The reason is not known. If rats of this weight are employed it is possible to determine the activity of an extract in about 14 days.

The weight increase attained at the end of the experiments (13/10/31) amounts to about 21·5 %. At this time the average weight of the experimental animals was steadily rising, whereas the weight-curve of the controls ran parallel with the abscissa. If the extract dose and experimental period are varied there is no doubt that much greater weight differences can be obtained than in the present series. It is possible that the vitamin content of the food plays an important rôle in this respect; this is a question that will be dealt with in a future publication. It will merely be pointed out here that the diet employed contains sufficient, but not a surplus of, vitamins. As the killing of the rats was spread over one month, it will be most correct to calculate their mean weight from weighings just after death; the weight difference was then 23·4 % (experimental animals: 355 g., controls: 288 g.).

Dry-matter determinations. The average dry matter percentage for the extract-treated rats (M_E) proved to be 36·5 ± 0·7, and for the controls (M_C) 38·6 ± 1·68. It will be observed that the individual variations in dry matter content are much greater for controls than for experimental animals. The great error in M_C has the effect that no importance is to be attached to the difference $M_C - M_E = 2{\cdot}1 \pm 1{\cdot}82$. This difficulty, of course, may be solved by increasing the number of controls. Fortunately, by means of Chanutin's investigations [1930], we are enabled to form a reliable idea as to the variability of the dry matter content in a random sample of rats comprising a sufficient number. In a series of 39 albino rats of both sexes Chanutin found an average dry matter content of 38·4 % (max. 45·7 and min. 32·8) and in another series of 45 similar animals 38·6 % (max. 42·2, min. 36·4), *i.e.* for both series a mean of 38·5. Unfortunately, the author does not give the mean error for this figure. For safety's sake we will therefore take it that the greatest deviation observed by him (7·2) corresponds with twice the standard deviation. The mean error of the mean (38·5) is then $7{\cdot}2/2\sqrt{84} = 0{\cdot}39$. There can scarcely be any doubt as to the justification for employing this author's experiments as a standard, *i.e.* substituting M_C (= 38·6 ± 1·68) with 38·5 ± 0·39, because in the first place these two figures are practically identical, and in the second place the greatest deviation in our control material amounts to 5·5, or $< 2\mu$ ($2 \times 3{\cdot}6$). The difference between the dry-matter contents of controls and experimental animals will then be:

$$(38{\cdot}5 - 36{\cdot}5) \pm \sqrt{0{\cdot}39^2 + 0{\cdot}7^2} = 2{\cdot}0 \pm 0{\cdot}8.$$

As the difference is > twice the mean error, it is probable that it is reliable, but by no means certain.

The total N determinations showed that the average N % in the experimental animals was 3·29 ± 0·49 and in the controls 3·16 ± 0·085. The difference is 0·13 ± 0·098, in other words insignificant from a statistical point of view, although of course this does not preclude the possibility that the test animals

nevertheless contain more N. The question calls for supplementary investigation.

With the collected data for dry matter and total N as a basis we are in a position to form an idea as to the nature of the growth that is induced by the extract employed. At the beginning of the experiments (3/2/1931) both experimental animals and controls weigh almost the same (Fig. 2). At the end (13/10–30/10/1931) the experimental animals on an average weigh 355 g. and the controls 288 g. with dry matter contents of 36·5% and 38·6 % respectively. Thus the increase of weight is 67 g., the increase of dry matter 18·4 g. and the percentage of dry matter in the increase 27·5. In normal rats weighing > 70 g. the percentage of dry matter is practically independent of the weight [*cf.* for instance Donaldson, 1915, p. 300]. Thus when a rat (> 70 g.) increases its weight by 67 g., it will, regardless of its momentary weight, increase its dry matter content by $\frac{67 \times 38{\cdot}6}{100} = 26$ g. Appraised in this manner we have thus to do with $\frac{18{\cdot}4 \times 100}{26}$ = about 71 % normal growth for our experimental animals. The N % in the experimental animals was 3·29 and in the controls 3·16. As no importance may be attached to this difference (see above), we will take it that experimental animals and controls contain an average of 3·23 % N. As the experimental animals weigh 67 g. more, they contain a N-surplus of $\frac{3{\cdot}23\,(355-288)}{100} = 2{\cdot}16$ g., which corresponds with 13·5 g. of protein.

It will be seen that the animals treated with extract not only add less dry matter than normal animals, but that their dry matter addition is of abnormal composition, having a much higher N content.

If the increase of weight induced by the anterior pituitary extract were exclusively due to retention of water, we should have found a dry matter content (M_E) in the experimental animals of $\frac{38{\cdot}5 \times 288}{355} = 31{\cdot}3$ % with a mean error (see above) of about $\pm$ 0·7. If the dry matter percentage of the controls (M_C) is put at 38·5 $\pm$ 0·39 (see above) the difference $M_C - M_E$ will be

$$(38{\cdot}5 - 31{\cdot}3) \pm \sqrt{0{\cdot}39^2 + 0{\cdot}7^2} = 7{\cdot}2 \pm 0{\cdot}8.$$

The difference actually found being 2·0 $\pm$ 0·8, it is out of the question that the induced weight increase is due exclusively to water. The same can be proved by the aid of the N analyses. If the weight difference were exclusively due to water, the N % in the experimental animals would fall to $\frac{3{\cdot}16 \times 288}{355} = 2{\cdot}56$. The difference between the average percentage in experimental animals and controls would then be:

$$(3{\cdot}16 - 2{\cdot}56) \pm \sqrt{0{\cdot}085^2 + 0{\cdot}049^2} = 0{\cdot}6 \pm 0{\cdot}098.$$

The difference between this figure and the difference found (0·13 $\pm$ 0·098) is 0·47 $\pm$ 0·14, *i.e.* statistically significant.

As was observed in the introduction, discontinuation of the injections causes an immediate loss of weight which amounts to about 35 % of the

difference attained. As the experimental animals have a higher water content than the controls, the obvious assumption is that it is this store of water which the animals get rid of when the treatment is discontinued. Therefore it will be of interest to calculate how much the animals can lose in weight by ridding themselves of the surplus quantity of liquid. The necessary data may be obtained from the curve in Fig. 1. It will be seen that on 22/12 the injected rats weigh 340 g. whereas the controls weigh 256 g. Discontinuation of the injections brings about a loss of weight of 32 g. If the water content of experimental animals is put at 63·5 % and of controls at 61·5 %, we obtain

$$\frac{\left(340 \times \frac{63{\cdot}5}{100} - X\right) 100}{340 - X} = 61{\cdot}5.$$

where X signifies the loss of weight through yielding up the surplus quantity of water. From the equation X is found to be about 18 g. Consequently, only about 60 % of the loss of weight actually found is due to elimination of the surplus water.

Summary.

In recent years various writers have published reports indicating that the increase of weight induced by bovine alkaline anterior pituitary extracts is principally the result of the retention of water. For the purpose of elucidating this problem a series of dry matter and total N analyses has been performed on extract-treated albino rats with corresponding controls. In the extract-treated animals there was found an average dry matter content of 36·5 %, in the controls 38·6 %. The difference is > twice the mean error. The average total N % in experimental animals and controls was 3·29 and 3·16 respectively. The difference is statistically insignificant. The figures show conclusively that the induced increase of weight is not exclusively due to retention of water; on the contrary, only a small part of the weight difference obtained seems to be a result of an abnormal accumulation of water. If the injections are stopped, the weight falls immediately, it is true, but the loss amounts to only about 35 % of the weight difference recorded. Only about 60 % of this loss of weight is explained by the excretion of surplus water. The nature of the growth induced by the extract employed may be characterised by the following figures. When an injected and a normal rat increase their weight by 100 g., these 100 g. will be of the following composition.

Injected rat: 72·5 g. water, 27·5 g. dry matter (consisting of 73 % protein and 27 % ash and fat).

Normal rat: 61·4 g. water, 38·6 g. dry matter (consisting of 52 % protein and 48 % ash and fat).

This work has been carried out with the support of the Carl V. Petersen Foundation. The extract employed was kindly prepared according to our directions by Miss Inger Lehmann, at the Lion Chemical Works. It is our pleasant duty to express our warmest thanks for the assistance thus given us.

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CXXI. APPLICATION OF THE SODIUM DIETHYLDITHIOCARBAMATE REACTION TO THE MICRO-COLORIMETRIC DETERMINATION OF COPPER IN ORGANIC SUBSTANCES.

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When an aqueous solution of sodium diethyldithiocarbamate is added to a solution containing copper the normal copper salt of diethyldithiocarbamic acid, which has a golden-brown colour, is formed. Callan and Henderson [1929], who discovered this reaction, applied their method with satisfactory results to the determination of copper in inorganic substances, but were not concerned with biological material.

Of the colorimetric methods which have been proposed for estimating the copper content of biological material the Biazzo [1926] method as developed by Elvehjem and Lindow [1929] has been most used. Apart from the fact that the accuracy of this method has been recently questioned [see Ansbacher, Remington and Culp, 1931; Walker, 1930; Drabkin and Waggoner, 1930] the carbamate reaction has, at the outset, certain important advantages over it, since the latter is at least ten times more sensitive and gives the same colour with copper irrespective of the p_H of the solution.

The work described in this paper was undertaken because we believed that to study further the rôle of copper in metabolism, and to clarify our present knowledge in this regard, more accurate methods were required for the determination of copper in organic materials.

Experimental.

The normal copper salt of diethyldithiocarbamic acid has a rather limited solubility in aqueous solution. If the amount of copper in the solution to be tested exceeds 0·015 mg. in 10 cc. the coloured copper salt tends to precipitate slowly and colorimetry becomes impossible. We have found that the coloured copper salt is much more soluble in amyl alcohol and may be extracted from aqueous solution by this solvent. The reaction is thereby made applicable over a much wider range of copper concentration. The ionisation of the copper salt is depressed in the organic solvent, thus intensifying the colour and so giving the reaction greater sensitivity.

To ascertain whether the intensity of the observed colour is proportional to the concentration of copper in the solution a series of proportionality tests covering three ranges of copper concentration was made. The results are plotted in Fig. 1 from data obtained as follows. Varying amounts of a standard copper

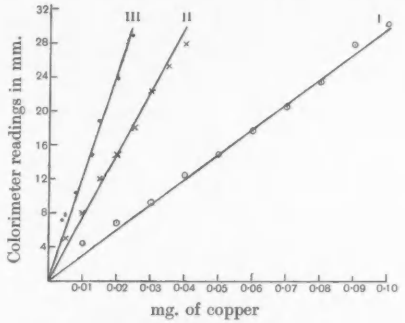

Fig. 1. Showing the proportionality between the amount of copper present and the intensity of the colour developed with sodium diethyldithiocarbamate.

Note: The values plotted represent the colorimeter reading in mm. of that solution chosen as a standard when the other test solutions were set at 15 mm.

	Range of copper concentrations mg.	Copper content of standard solution mg.
Curve I	0·01 –0·10	0·05
Curve II	0·005–0·04	0·02
Curve III	0·004–0·024	0·012

The diagonal lines represent the theoretical proportionality between copper present and colour produced.

solution were measured into 25 cc. glass-stoppered measuring cylinders and all diluted to the same volume (10 cc.)[1] with water. To each cylinder were added 1 drop 40 % NaOH, 5 cc. amyl alcohol (carefully measured) and finally 0·5 cc. of a 2 % aqueous solution of the carbamate reagent[2]. The cylinders were stoppered and shaken, not too vigorously, for about one minute. The amyl alcohol layer, which separated very quickly and contained the whole of the coloured copper salt, was removed by means of a pipette and filtered into a clean dry test-tube through a small 2·5 cm. filter. This filtration, which removes the small amount of water suspended in the amyl alcohol, was adopted as a

[1] In all the experiments discussed in this paper special precautions were taken to preclude any possibility of copper contamination. For preparing the reagents, rinsing glassware, *etc.*, only water redistilled from glass was used. All the reagents were tested for copper. NaOH prepared from sodium which contained no copper was always used. Merck's highest purity amyl alcohol was employed. The sodium salt of diethyldithiocarbamic acid was obtained from the Eastman Kodak Co. When incineration was employed to destroy organic matter only "vitreosil" dishes or porcelain dishes specially treated to ensure absence of copper [Elvehjem and Lindow, 1929] were used.

[2] An aqueous solution of sodium diethyldithiocarbamate keeps fairly well when stored in a dark-coloured bottle. A precipitate forms in a few weeks. After filtering the reagent may still be used.

time-saving measure. The amyl alcohol layer will become perfectly clear if allowed to stand for about 30 minutes. In the third series of proportionality tests the total volume of each solution was 5 cc. instead of 10 cc., and only 3 cc. of amyl alcohol were used for extraction. The colorimetry was carried out in a Duboscq colorimeter using micro-cups.

The depth of colour is directly proportional to the amount of the coloured copper salt present provided the difference between the amounts of copper in the standard solution and the test solution is not too great (Fig. 1). A standard containing 0·005 mg. copper was found to cover the range from 0·002 mg. to 0·010 mg. of copper very satisfactorily. The solutions are stable towards light for at least 12 hours. It is important that the amyl alcohol used should be of the highest purity. The colour fades slowly in the technical grade of solvent.

Extractions with chloroform and with carbon tetrachloride have been tried but were found to be unsatisfactory.

Callan and Henderson [1929] investigated the reaction of the carbamate reagent with the more common metals. Iron which gives a brown colour with the reagent is the only element present in the ash of biological material which would interfere with the determination of copper. Less than 0·05 mg. Fe gives no colour. The intensity of the colour given by 0·08 mg. Fe is approximately equal to that given by 0·005 mg. Cu. Because of the difference in colour the two solutions can only be very roughly matched.

According to Callan and Henderson [1929] iron can be completely removed as ferric hydroxide without loss of copper. This procedure has been found by Ansbacher *et al.* [1931] to give copper values which are too high. The present author [McFarlane *et al.*, 1930], in determining the copper content of hen's eggs, used double precipitation with NH_4OH to remove iron and found the filtrate to contain all the copper free from iron.

The estimation of copper in solutions containing relatively large amounts of iron when sodium pyrophosphate is present.

When sodium pyrophosphate is added to a solution containing copper and iron, $Fe_4(P_2O_7)_3$ and $Cu_2P_2O_7$ are formed. The former is an un-ionised compound and does not react with such ions as CNS^- [Warburg, 1927] nor does it react, as we have found, with the carbamate reagent. The pyrophosphate does not affect the reactivity of the copper. Drabkin and Waggoner [1930] recommend a 4 % solution of sodium pyrophosphate to remove the iron when determining copper by the Elvehjem and Lindow procedure. In a later paper Elvehjem and Hart [1931] claim that in using $Na_4P_2O_7$ for this purpose accurate results are obtained only when certain precautions as to p_H and temperature are taken.

To a series of tubes all containing the same amount of copper, but with increasing amounts of ferric chloride, varying amounts of 4 % $Na_4P_2O_7$ were added. The solutions were then made alkaline by adding 1 cc. conc. NH_4OH and heated in a water-bath at 80° for 10 minutes. No precipitate of ferric pyrophosphate was formed under these conditions. The colorimetry was com-

pleted as before, using one of the solutions as a standard. Against this the relative intensities of colour of the others were determined.

Table I. *The effect of sodium pyrophosphate on the recovery of copper from solutions containing 0·02 mg. Cu to which varying amounts of iron have been added.*

	Amount of iron added mg.	Amount of 4 % $Na_4P_2O_7$ added cc.	Colorimeter reading mm.*
1.	Nil	1·0	20·0
2.	Nil	3·0	20·8
3.	0·35	Nil	reddish-brown precipitate
4.	0·35	1·0	20·4
5.	0·70	1·0	21·0
6.	1·05	1·0	reddish-brown precipitate
7.	1·05	3·0	21·3
8.	1·40	3·0	21·8
9.	1·75	3·0	22·0

* The colorimeter readings are those of solution No. 1 as standard when adjusted to match the other test solutions which were always set at 20 mm.

We may thus conclude from Table I that copper can be accurately estimated in alkaline pyrophosphate solution in the presence of relatively large amounts of iron. The presence of traces of copper in the pyrophosphate and ferric chloride solutions was responsible for the variations in the colorimeter readings.

The determination of copper in crude glutamic acid[1].

Our first analyses were done on crude glutamic acid because of our interest in this product at the time. A qualitative examination had shown that it contained a very considerable amount of copper and iron.

Samples were incinerated in a muffle furnace at 380–400° for at least 12 hours. We have found that to recover copper quantitatively from the ash of organic materials the same careful control of the temperature of ashing as that recommended by Peters [1912] for the estimation of iron is necessary. The dishes were cooled and the ash was moistened with 1–2 cc. conc. HNO_3. The HNO_3 was driven off by heating over a small flame and the dishes were replaced in the muffle for a short time. The ash should be perfectly white or, if much iron is present, reddish in colour. If unoxidised carbon is still present the wet incineration with HNO_3 should be repeated. The ash is completely soluble in hot 6*N* HCl.

The results of copper determinations on crude glutamic acid by various procedures are recorded in Table II. With the larger samples of glutamic acid suitable aliquots of the ash solution were taken for the analysis. Where $Na_4P_2O_7$ was used to remove the iron the procedure was the same as that already described. To remove iron as $Fe(OH)_3$ an excess of concentrated NH_4OH was added to an aliquot of the ash solution in a 15 cc. centrifuge-tube. After heating on a boiling water-bath for 15 minutes the solution was centrifuged and the clear supernatant liquid decanted into a 25 cc. glass-stoppered cylinder.

[1] *d*-Glutamic acid (practical). Eastman Kodak Co.

Table II. *The copper content of crude glutamic acid as determined by different procedures.*

Wt. of glutamic acid	Copper added mg.	Method of destroying organic matter	Method of removing Fe	Colorimetric method used	% Cu in crude glutamic acid
10 g.		Dry ashing	Aliquot 1 $Na_4P_2O_7$	Carbamate reagent	0·0173
			" 2 "	" "	0·0167
			" 3 "	Elvehjem and Hart [1931]	0·013
10 g.		" "	Aliquot 1 NH_4OH	Carbamate reagent	0·0159
			" 2 "	" "	0·0168
			" 3 "	Elvehjem and Hart	0·011
2 g.	0·10	" "	Aliquot 1 $Na_4P_2O_7$	Carbamate reagent	0·0187*
			" 2 "	" "	0·0174
			" 3 "	Elvehjem and Hart	0·014
0·5 g.		" "	$Na_4P_2O_7$	Carbamate reagent	0·0174
0·5 g.	0·01	" "	"	" "	0·0177*
0·5 g.		H_2SO_4-$HClO_4$ digestion	"	" "	0·0185
0·5 g.		" "	"	" "	0·0178
0·10 g.		Dry ashing	"	" "	0·0201
					0·0179

* The amount of copper added was subtracted from the amount of copper found before calculating the copper content of the crude glutamic acid.

The $Fe(OH)_3$ precipitate was dissolved in the minimum amount of 6*N* HCl (with the aid of heat) and $Fe(OH)_3$ reprecipitated as before. The copper content of the combined supernatant liquids was then determined as described above.

Preparation of the standard copper solution. 0·3928 g. pure $CuSO_4$, $5H_2O$ was dissolved in water. A small amount of dilute H_2SO_4 was added to prevent the formation of basic cupric sulphate by hydrolysis. The solution was then diluted to 1 litre (1 cc. = 0·10 mg. Cu). In the majority of the determinations 1 cc. of a standard solution containing 0·01 mg. Cu per cc. was used. In preparing this standard the same reagents were added in the same amounts as those used in preparing the solution being analysed, *i.e.*, 6*N* HCl and $Na_4P_2O_7$ solution or concentrated NH_4OH.

The destruction of organic matter by wet incineration was carried out in a micro-Kjeldahl flask. 2 cc. concentrated H_2SO_4 and 1 cc. 60 % $HClO_4$ were added and the solution was heated over a micro-burner until only a faint yellow colour persisted and white fumes ceased to be evolved.

The values obtained for the copper content of crude glutamic acid (Table II) were in good agreement, whether the organic matter was destroyed by dry or by wet incineration. The removal of iron as $Fe(OH)_3$ or as $Fe_4(P_2O_7)_3$ made no appreciable difference to the results. The values obtained, using the method of Elvehjem and Hart [1931], were considerably lower than those obtained when the same sample was analysed by means of the carbamate reagent.

The determination of copper in blood.

The copper content of blood or serum according to various authors is given in Table III. From the results of these analyses it appears that either the copper contents of the bloods of different species and of individuals within

the same species are subject to considerable variations or that the analytical methods are inaccurate.

Table III.

	Mg. copper per 100 cc.
Whole or defibrinated horse-blood [Elvehjem, Steenbock and Hart, 1929, 1].	0·058*
" " " [Grendel, 1930].	0·147
Three samples of whole human blood [Schönheimer and Oshima, 1929].	0·113 to 0·114*
" " " [Grendel, 1930].	0·075
Horse-serum [Abderhalden and Möller, 1928].	0·19
Cow's serum [McHargue, Healy and Hill, 1928].	0·33
Ox-blood [Grendel, 1930].	0·076

* Same colorimetric method used.

Samples of blood (3–10 cc.) were evaporated to dryness on a water-bath, a small glass rod being used for stirring. The dried blood without removing the stirring rod was ashed at 400° as already described. The ash was dissolved in 2–5 cc. 6*N* HCl, with the aid of heat, the amount of HCl used depending upon the amount of blood taken. The solution of the ash was transferred to a 25 cc. glass-stoppered cylinder with the aid of a few cc. of water. 1–5 cc. saturated sodium pyrophosphate (about 5·4 g. $Na_4P_2O_7$, 10 H_2O in 100 cc. cold water) were added, the amount again depending upon the quantity of blood being analysed. For 5 cc. of blood 3 cc. saturated solution of $Na_4P_2O_7$ were used. Concentrated NH_4OH was added from a burette until the solution was just alkaline to litmus. The cylinders were placed in a water-bath (80°) for about 15 minutes and then cooled. With the larger samples of blood (*i.e.*, about 10 cc.) a slight white precipitate, presumably calcium phosphate and $Mg_2P_2O_7$, is formed but does not interfere with the colorimetric determination. The amount of copper present was finally determined, as already described, using the carbamate reagent and extracting with amyl alcohol. For 5 cc. of blood a suitable standard contained 0·01 mg. Cu to which had been added the same amount of $Na_4P_2O_7$ and NH_4OH and which had been diluted to the same volume as the test solution.

When the iron was removed as $Fe(OH)_3$ the above procedure was modified in the same way as that described for glutamic acid.

Results.

1. *Whole ox-blood.*

	Mg. copper in 100 cc. of blood
A. 5 cc. blood—ash oxidised with HNO_3	
(1) Iron removed with NH_4OH	0·185
(2) " " " "	0·187
(3) " " " $Na_4P_2O_7$	0·187
B. 5 cc. blood—ash not oxidised with HNO_3	
(1) Iron removed with NH_4OH	0·226
(2) " " " "	0·208
(3) " " " $Na_4P_2O_7$	0·191
C. 3 cc. blood—ash oxidised with HNO_3	
(1) Iron removed with NH_4OH	0·172
(2) " " " $Na_4P_2O_7$	0·190

2. *Defibrinated ox-blood.*

Iron removed as $Fe_4(P_2O_7)_3$ and the ash oxidised with HNO_3.

Amount of blood taken for analysis cc.	Amount of copper added mg.	Copper content of the blood calc. after subtracting the amount of copper added mg. per 100 cc.
2·0	0·004	0·221
2·0	0·008	0·214
5·0	Nil	0·202
5·0	0 006	0 194
5·0	0·010	0·210
3·0	Nil	0·190

3. *Whole pig-blood.*

10 cc. blood taken for analysis. Ash oxidised with conc. HNO_3	Mg. copper in 100 cc. blood
(1) Iron removed with NH_4OH	0·163
(2) „ „ „ $Na_4P_2O_7$	0·180

4. *Whole human blood.*

Ash oxidised with HNO_3. Iron removed with $Na_4P_2O_7$.

(1) 10 cc. blood from one individual	0·185
(2) 10 cc. „ „ another individual	0·170
(3) 5 cc. „ (composite sample)	0·181
(4) 5 cc. „ „ „	0·210

Note. The composite samples were from oxalated blood received by this laboratory for routine analysis.

5. *Defibrinated ox-blood: sulphuric-perchloric acid digestion.*

To 3 cc. of blood in a 100 cc. Kjeldahl flask, 2 cc. conc. H_2SO_4 and 1 cc. 60 % $HClO_4$ were added and digestion continued until foaming ceased. Two further 1 cc. portions of 60 % $HClO_4$ were required to complete the incineration, when white fumes were no longer evolved and the light yellow-coloured sulphuric acid digest boiled very slowly. The copper contents of three samples of the same blood were determined with the following results. $Na_4P_2O_7$ was used to remove the iron.

(1) 0·215 mg. copper per 100 cc. blood
(2) 0·145 „ „ „ „ „
(3) 0·165 „ „ „ „ „

The results by this method of incineration while less accurate are of the same order of magnitude as those obtained by dry ashing. Difficulties in manipulation, at least so far as blood is concerned, would not warrant the substitution of acid incineration for dry ashing even if more consistent results were obtained after further study of the wet incineration method.

The copper content of blood can be accurately estimated by means of the carbamate reagent, particularly if the blood is ashed, the ash oxidised with HNO_3 and the iron removed as $Fe_4(P_2O_7)_3$.

The determination of copper in crystalline haemoglobin.

Elvehjem, Steenbock and Hart [1929, 1] have recorded the copper contents of different crystalline preparations of haemoglobin as 3·4 and 1·9 mg. Cu per 100 g. respectively for two samples from horse-blood, and 1·3 and 1·7 mg. Cu per 100 g. for two different preparations from rat-blood. These values appear to be very high, especially as, in the same paper, they record the copper content of the corpuscle fraction of horse-blood as only 0·043 mg. Cu per 100 cc. of blood. These analyses were made in an endeavour to answer the question "Is copper a normal constituent of the haemoglobin molecule?" Their conclusion, which was in the negative, was not based on their experimental findings but on the fact that a positive result was incompatible with the accepted molecular weight of haemoglobin. It is of interest in this connection to record here the copper content of a sample of crystalline haemoglobin, of German preparation but of unknown origin. This sample contained only 0·0057 mg. Cu per g. of haemoglobin as determined by our method of analyses.

The determination of copper in cow's milk.

It was with the realisation that there was a particular need for an accurate method of determining the minute amount of copper in cow's milk that we first undertook the investigations recorded in this paper. Perhaps the best evidence that existing methods for determining the copper content of milk are of doubtful accuracy is to be found in the numerous papers on the rôle of copper as an essential supplement to inorganic iron in curing nutritional (milk) anaemia in rats. We believe that much of the confusion which exists in this regard (see Elvehjem and Hart [1932] for brief discussion) is the inevitable result of carrying out such feeding experiments without an accurate knowledge of the copper content of the milk.

According to various authors the copper content of cow's milk, collected with special precautions, is:

	Mg. copper per litre
*Elvehjem, Steenbock and Hart [1929, 2]	0·15
*Drabkin and Waggoner [1930]	0·25
*Beard and Myers [1931]	0·44
**Supplee and Bellis [1922]	0·2 to 0·8
**Quam and Hellwig [1928]	0·26 to 0·52
Keil and Nelson [1931]	0·24

*Determined by the method of Elvehjem and Lindow [1929].
**Potassium ethyl xanthate method.

The value recorded by Keil and Nelson is "as determined by the sodium diethyldithiocarbamate method." Since these authors made no further comment it was to be assumed that the accuracy of this method had been established. We could find, in the literature, no earlier reference to the use of this reagent for determining the copper content of milk.

The estimation of copper in milk presents certain difficulties which are to some extent peculiar to this product. The minute amount of copper present

makes it necessary to use a large sample for the analyses. The solution of the milk-ash then contains a relatively large amount of calcium phosphate. The methods of removing iron, already discussed, are not applicable, as the copper cannot be quantitatively recovered from the voluminous precipitate of calcium phosphate which comes down in alkaline solution.

Precipitation of minute quantities of copper with hydrogen sulphide. The majority of the more recent determinations of the copper content of milk have been made by the method of Elvehjem and Lindow [1929]. By this procedure the copper (0·03 to 0·04 mg. from 250 cc. milk) is precipitated by H_2S in a total volume of 200 cc. 0·25*N* HCl. This large dilution of the milk-ash before H_2S precipitation appeared to us to involve an unnecessary danger of incomplete precipitation of cupric sulphide. These conditions of H_2S precipitation were probably adopted by Elvehjem and Lindow to avoid the simultaneous precipitation of much free sulphur which interfered with their final colorimetry. According to Ansbacher *et al.* [1931], the precipitation of small amounts of copper by H_2S is only quantitative when the concentration of acid is such that some sulphur is precipitated to adsorb and carry down the cupric sulphide. According to Walker [1930], the oxidation of cupric sulphide by nitric acid in the presence of filter-paper gives results for copper which are too high.

Our first experiments were conducted with an inorganic solution the composition of which approximated to that of a solution of milk-ash so far as the elements Fe, Cu, Ca and P were concerned. We first studied the influence of acidity and dilution on the recovery of the copper from this solution. The copper solutions prepared (see Table IV) were heated to 80° in 100 cc. glass-

Table IV.

	Amount of copper present mg.	Amount of 6*N* HCl added cc.	Total volume at H_2S precipitation cc.	Normality	Amount of copper recovered mg.
1.	0·02	5·0	100	0·25	0·011
2.	0·02	5·0	50	0·50	0·021
3.	0·02	5·0	10	2·5	0·020
4.	0·01	2·5	50	0·25	0·008
5.	0·01	2·5	10	1·25	0·012

stoppered Erlenmeyer flasks and while cooling were saturated with H_2S for 15 minutes. The flasks were stoppered and set aside for 24 hours. The solutions, without heating, were resaturated with H_2S for 15 minutes. To avoid the use of filter-paper, micro-Jena glass sintered filters set in test-tubes with a side-arm were used for filtration of the cupric sulphide under suction. The precipitate was washed on the filter with a few cc. of H_2S water which had just been used to rinse the Erlenmeyer flasks. After removing the clear filtrate the filter was replaced in the test-tube. 2 cc. 10 % H_2SO_4 and 1 cc. saturated bromine water were measured into the Erlenmeyer flask (to oxidise any CuS still remaining) and then transferred to the Jena filter and allowed to stand 15 minutes without

suction. A small glass rod was used to stir up the precipitate. By applying suction the solution was drawn into the test-tube. Four 1 cc. portions of water were used to rinse the Erlenmeyer flask and filter. A small amount of free sulphur is left on the filter. The CuS is completely oxidised and all the copper is in the filtrate.

The filter was removed and the excess bromine in the filtrate driven off by carefully bringing the contents of the test-tube to the boil over a small flame. After cooling, 0·75 cc. 40 % NaOH was added, *i.e.*, the amount requisite to make the solution just alkaline to phenolphthalein as previously determined by blank titration. The colorimetry with the carbamate reagent was carried out in 25 cc. glass-stoppered measuring cylinders as previously described.

The standard (usually containing 0·01 mg. copper) was prepared by measuring into a test-tube an aliquot of the standard copper solution. 2 cc. 10 % H_2SO_4 and 1 cc. saturated bromine water were added and the excess bromine was driven off by boiling. After transferring the solution to a 25 cc. glass-stoppered cylinder the preparation was completed in the same manner as described for the test solution. It was decided from these results (Table IV) that if the milk-ash were dissolved in 10 cc. 6*N* HCl and the solution diluted to not more than 50 cc. quantitative precipitation of copper by H_2S would be obtained.

The milk was obtained by milking directly into glass containers. Samples of 100–300 cc. were evaporated to dryness on a water-bath. The residue was ashed in a muffle furnace (for method of ashing see under glutamic acid). The ash was dissolved in 10 cc. 6*N* HCl and the solution transferred to a 100 cc. glass-stoppered Erlenmeyer flask with the aid of 40 cc. water. The precipitation of copper as cupric sulphide, the filtration and oxidation of this precipitate and the final colorimetric determination of copper by the carbamate reagent were carried out as described above. The three samples of milk analysed were collected over a period of two months.

Sample 1		Sample 2		Sample 3	
cc. milk analysed	Copper content mg. per litre	cc. milk analysed	Copper content mg. per litre	cc. milk analysed	Copper content mg. per litre
250	0·092	250	0·068	300	0·072
250	0·100	250	0·067	250	0·077
200	0·097	150	0·049	200	0·071
100	0·068	*300	0·120	**250	0·080
*300	0·144	—	—	*300	0·115

* Determined by the method of Elvehjem and Lindow [1929].

** The CuS precipitate was oxidised on the Jena filter with 2 cc. hot concentrated HNO_3 instead of the Br_2-H_2SO_4 solution. The filtrate was neutralised with 3·15 cc. 40 % NaOH and the copper determination carried out with the carbamate reagent.

We can thus conclude from these results that the copper content of milk can be accurately determined by the method described provided the amount of milk taken for analyses is not less than 200 cc. In addition to the results recorded a number of analyses using 75–150 cc. of milk have given low results. It will

also be observed that with the same samples of milk the method of Elvehjem and Lindow gave considerably higher results than were obtained by the carbamate method. The oxidation of CuS in the cold by bromine in sulphuric acid solution has proved extremely satisfactory. Hot conc. HNO_3 gives the same results, provided the standard copper solution used for colorimetric comparison contains an equal amount of HNO_3. The intensity of colour with the carbamate reagent is considerably less when HNO_3 has been used as the oxidising agent instead of the bromine-sulphuric acid solution.

The determination of copper in rat-livers.

Dr R. C. Hamilton of this laboratory in studying certain aspects of copper metabolism has analysed a large number of rat-livers for copper. With a few minor modifications the procedure recommended in this paper for estimating the copper content of blood was used. The samples of liver, about 1 g., were ashed and the ash was dissolved in 5 cc. 6*N* HCl with the aid of heat. 3 cc. saturated solution of $Na_4P_2O_7$ were used to remove the iron and 3 cc. amyl alcohol to extract the coloured copper salt.

	Weight of liver taken for analysis g.	mg. of copper per 100 g. of tissue—calculated on A *Dry weight of liver	B Wet weight of liver
1. Normal adult rat (male)	1·6135	2·18	0·59
	1·3085	1·99	0·56
2. Normal adult rat (male)	1·4680	2·09	0·62
	1·8233	1·94	0·57
3. Normal adult rat (female)	1·2798	2·54	0·69
	1·0745	2·87	0·74
	0·9602	2·71	0·71

* The portion of liver was dried at 100° for 12 hours.

These results in the hands of another worker are a good confirmation of the consistency with which the copper content of biological materials can be determined by this method. Accurate copper determinations are possible using as little as 0·25 g. of liver.

Summary.

1. An examination of the reaction between copper and sodium diethyldithiocarbamate (Callan and Henderson) has revealed certain points of importance in the quantitative application of this reaction:

A. The golden-brown copper salt formed is much more soluble in amyl alcohol than it is in water. The coloured copper salt can be rapidly and quantitatively extracted from aqueous solution by amyl alcohol. The reaction may then be applied over a much wider range of copper concentration.

B. The golden-brown colour is intensified in the organic solvent. The sensitivity of the reaction is thus increased.

C. The depth of colour is directly proportional to the amount of copper present provided the range of copper concentrations is not too great.

D. In alkaline solution ferric pyrophosphate does not give the reaction but the reactivity of the copper is unimpaired. In alkaline pyrophosphate solution copper may be determined in the presence of iron.

E. The oxidation of cupric sulphide in the cold with saturated bromine water and 10 % sulphuric acid does not interfere with the subsequent application of the colour reaction.

2. These observations have been utilised in developing procedures by means of which copper may be accurately estimated in biological materials. Blood, milk, liver, crude glutamic acid and crystalline haemoglobin have been analysed for copper in the course of these investigations.

I desire in conclusion to express my thanks to Prof. G. Hunter for his constant advice and help in this work. I am also indebted to Dr R. C. Hamilton for permitting me to include the results of some of his copper determinations on rat-livers.

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CXXII. THE DETERMINATION OF IRON IN ORGANIC SUBSTANCES.

I. MICRO-COLORIMETRY AS FERRIC THIOCYANATE.

II. POTENTIOMETRIC TITRATION WITH TITANOUS SULPHATE.

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In certain experiments on iron metabolism, involving the iron-containing protein of egg-yolk, lecitho-vitellin, we planned to make a large number of iron estimations. To conserve our small supply of protein, the preparation of which had involved considerable time and labour, we desired to cut down the size of the sample analysed to the smallest amount on which an accurate iron determination could be made. The author and colleagues [1930] had already found that the iron content of egg-yolk protein could be accurately determined by the method of Kennedy [1927]. The samples then analysed contained about 0·05 mg. iron, an amount somewhat greater than that which we now wished to estimate.

The results of a critical study of Kennedy's procedure as the basis of a micro-colorimetric method are recorded in the first part of this paper. The second part is concerned with the determination of small amounts of iron in biological materials by potentiometric titration with titanous sulphate.

I. The determination of minute amounts of iron by the method of Kennedy.

Of the recent methods recommended for the oxidation of organic matter preliminary to estimation of iron in organic materials, [Willstätter, 1920; Fowweather, 1926; Kennedy, 1927; Smirk, 1927; Wong, 1928; Elvehjem, 1930] we have found the sulphuric-perchloric acid method of Kennedy to be the most rapid and most convenient. Our adaptation of this method is described later. With some materials "ashing" is the only satisfactory procedure.

The conditions for maximum sensitivity of the ferric thiocyanate reaction when applied to a sulphuric-perchloric acid digest.

Many of the conflicting data on iron estimations [see Nottbohm and Weisswange, 1912; Stugart, 1931; Lásztó, 1931] appear to have resulted from a lack of appreciation of the complexity of the reaction between ferric salts and thiocyanates.

I. *The effect of reduction.* Philip and Bramley [1913] have studied the reduction which occurs in a ferric thiocyanate solution on standing with formaldehyde

$$8Fe(CNS)_3 + CH_2O \rightleftharpoons 8Fe(CNS)_2 + 7HCNS + CO_2 + NH_3(?) + H_2SO_4.$$

This reduction, due to the oxidising power of $Fe^{\equiv}$ can obviously be retarded by increasing the acidity and the thiocyanate concentration. The reduction in the presence of H_2SO_4 is about twice that in the presence of HCl. When amyl alcohol is used to extract the ferric thiocyanate from aqueous solution (as recommended by Kennedy) it is improbable that reduction can take place to any great extent. However, when determining very small amounts of iron in sulphuric acid solution, and even when using amyl alcohol, the possibility of reduction of ferric thiocyanate (bleaching of the red colour) should not be overlooked.

II. *The effect of acidity.* If the p_H is low, the number of undissociated molecules of ferric thiocyanate, and hence the depth of red colour, may be decreased by the union of H^+ with CNS^-, to form comparatively slightly ionised thiocyanic acid.

Solution No.	Amount of 40 % NaOH solution added cc.	Reading of standard (Solution No. 1) when other test solutions were set at 15 mm.
1	3·0	—
2	2·5	15·1
3	2·25	16·4
4	2·0	17·5
5	1·75	15·7
6	1·5	14·8
7	1·0	14·1
8	0·5	13·8
9	Nil	13·8

A solution was prepared which contained in 4 cc. 0·02 mg. ferric iron, 1 cc. concentrated sulphuric acid, 0·5 cc. 60 % perchloric acid and 1 drop conc. nitric acid. Into each of eight 10 cc. glass-stoppered measuring cylinders were measured 4 cc. of this iron-containing solution and varying amounts of a 40 % sodium hydroxide solution[1]. All the solutions were brought to the same volume and finally 1 cc. 40 % potassium thiocyanate solution and 3 cc. pure amyl alcohol were added to each. The tubes were shaken, and after standing a few minutes, until the aqueous layer had become colourless, as much as possible of the amyl alcohol layer was transferred to small filters (2·5 cm.). This filtration

[1] Sodium hydroxide prepared from sodium and found to contain no iron.

through paper removes finely divided particles of water dispersed in the amyl alcohol, and permits excellent colorimetry. Without such treatment, the solution of ferric thiocyanate in amyl alcohol always appears very slightly turbid. A colorimeter of the Duboscq type with micro-cups was used.

The maximum intensity of colour was obtained with solution **No. 4**, which was approximately 1·6 *N* acid. According to Lachs and Friedenthal [1911] the number of undissociated molecules of ferric thiocyanate reaches a maximum in 2 *N* HCl.

III. *The effect of thiocyanate concentration.* Solutions containing varying amounts of potassium thiocyanate were prepared. A series of iron solutions was prepared, each having the same composition as Solution No. 4 in the pre-

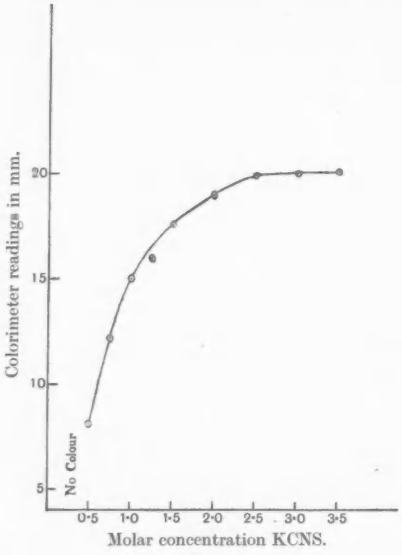

Fig. 1.

ceding experiment. Potassium thiocyanate in varying dilutions was added to the iron solution, 1 cc. to each cylinder. The colorimetric determination was carried out as already described.

It may be concluded from the results shown in Fig. 1 that the maximum intensity of colour with equal amounts of iron (0·02 mg. in 1·6 *N* acid) is obtained with 1 cc. 3·5 to 4·0 *M* potassium thiocyanate solution. This concentration is obtained with approximately 1 cc. 40 % potassium thiocyanate solution. The shade of colour changes slightly towards a brownish-red with increasing amounts of potassium thiocyanate.

IV. *The effect of phosphate.* The lecitho-vitellin which we prepared contained 0·59 % phosphorus. When determining iron as ferric thiocyanate in the ash of biological materials, Elvehjem and Hart [1926] have given particular attention

to the interfering action of large amounts of phosphate. According to Leeper [1930], the interfering action of orthophosphate has been overemphasised, the effect being primarily due to pyrophosphates, which are formed only on ashing at high temperatures. According to Kennedy and to Elvehjem and Hart, pyrophosphates are not formed during sulphuric-perchloric acid digestion. Leeper has recorded that high concentrations of phosphate do, however, "turn the red colour of ferric thiocyanate progressively towards a straw yellow" which cannot be matched in tint with phosphate-free solutions. This effect should be minimised by using the high concentration of thiocyanate which we have found necessary for the full development of the red colour. Quantitative determinations of iron in varying quantities of lecitho-vitellin show that the presence of phosphate has no effect on the results obtained (see Table I).

V. *The effect of varying amounts of iron (0·0025–0·025 mg.) on the intensity of the red colour.* A series of iron solutions was prepared, each solution containing the same amount of sulphuric acid and perchloric acid. The reaction in each

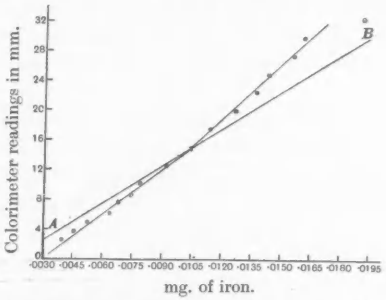

Fig. 2. Line A–B = Theoretical proportionality.

case was adjusted to approximately 1·6 N acid with 40 % sodium hydroxide. To 5 cc. of each dilution in a 10 cc. glass-stoppered measuring cylinder were added 1 cc. 40 % potassium thiocyanate solution and 3 cc. amyl alcohol.

The colorimetry was carried out as follows. The solution chosen as standard, which contained 0·0105 mg. iron, was always adjusted to match the other test solutions, which were set at 15 mm. The advantage of this procedure, as pointed out by Hunter and Eagles [1925] is that any divergence from proportionality is more easily seen when the graph is a straight line instead of a logarithmic curve. In this way, the colorimeter readings are also shown as increasing with increasing concentration of the coloured substance.

Within the range of iron concentrations employed, the intensity of the red colour is not directly proportional to the amount of iron present (see Fig. 2). When applying the thiocyanate reaction to the estimation of minute amounts of iron (0·0025 to 0·025 mg.) under the conditions just described, a correction must be made for this deviation from Beer's Law. In estimating the iron content of lecitho-vitellin, this correction was made directly from Fig. 2.

The estimation of iron in lecitho-vitellin.

Samples of lecitho-vitellin (18·65 to 127 mg.) in small glass tubes, were weighed on a micro-balance, and transferred to micro-Kjeldahl flasks, and the weight of the sample was obtained by reweighing the tube. 1 cc. of concentrated sulphuric acid, 0·5 cc. 60 % perchloric acid, and a glass bead were added, and the digestion was carried out over a micro-burner. Towards the completion of oxidation (10 to 15 minutes) the digest becomes almost colourless and boils very slowly. This is immediately followed by sudden ebullition and the appearance of a fairly intense yellow colour in the solution. The evolution of dense white fumes at this stage marks the end of the digestion. After cooling, 1 drop of concentrated nitric was added and the digest again heated to boiling to drive off the excess nitric acid.

The digest was transferred to a 10 cc. volumetric flask and the reaction adjusted so that when diluted to the 10 cc. mark the solution was between 1·5 and 2·0 *N* acid[1]. A 4 cc. aliquot was measured into a 10 cc. glass-stoppered measuring cylinder, and the colorimetry carried out as already described, using 1 cc. 40 % potassium thiocyanate solution and extracting the ferric thiocyanate with 3 cc. amyl alcohol.

The standard iron solution was prepared so as to contain the same reagents in the same amount as the unknown test solution. The iron content of the same sample of lecitho-vitellin was also determined by the method of Elvehjem [1930] and by the unmodified procedure of Kennedy [1927].

Table I.

Method of analysis	Weight of lecitho-vitellin mg.	Iron content of a 4 cc. aliquot of digest after subtracting the graph correction mg.	% iron in lecitho-vitellin
Modified Kennedy procedure	127·23	0·022	0·044
,,	121·70	0·022	0·045
,,	87·27	0·015	0·044
,,	74·23	0·013	0·045
,,	68·41	0·014	0·047
,,	49·38	0·009	0·045
,,	38·31	0·007	0·046
,,	27·00	0·007	0·046
,,	18·65	0·004	0·050
Unmodified Kennedy procedure	316·00	—	0·041
Elvehjem ashing method [1930]	79·33	—	0·054
,,	87·31	—	0·051
,,	102·74	—	0·059

[1] This dilution permits duplicate colorimetric determinations to be made on each sample. In later analyses the digest was transferred directly to a 10 cc. measuring cylinder with the aid of three 1 cc. portions of water. 2 cc. of 40 % sodium hydroxide solution were carefully added from a burette, and after cooling the determination was completed as above.

The values obtained for the iron content of lecitho-vitellin were in extremely good agreement when the final colorimetric determination was made with amounts of iron from 0·007 to 0·022 mg. The analysis of a larger sample of the protein by the unmodified procedure of Kennedy gave a slightly lower value. The method of Elvehjem gave figures which were not only higher than those obtained by acid digestion but were also more variable. We are in complete agreement with Stugart [1931] that any method of determining iron, such as that of Elvehjem, which involves boiling with alkali for one hour in a glass container, invites iron contamination as a result of the solvent action of the hot alkali on the glass.

II. The determination of small amounts of iron in an acid digest of an organic substance by potentiometric titration with titanous sulphate.

The rapid and accurate method of determining ferrous and ferric iron by titanium titration described by Knecht and Hibbert [1903] has been applied to biological materials by Jahn [1911], Peters [1912], Pincussen and Roman [1931] and Lásztó [1931]. In modifying the procedure Jahn used Neumann's wet incineration with nitric acid to destroy organic matter. The nitric acid was removed by boiling with sulphuric acid and the titration finally made with $N/250$ to $N/500$ titanous chloride at 10°, using potassium thiocyanate indicator. Peters applied the titration in essentially the same way to a solution of the ash of the organic substance. Barcroft [1914] considered the method to be highly accurate and simple, while Murray [1924], disagreeing so far as the simplicity is concerned, has recommended micro-titration with permanganate. According to Lásztó, the iron content of blood cannot be determined with sufficient accuracy by the titanium-salt process of Jahn.

Recently Pincussen and Roman [1931] have developed a method, based on titanium-salt titration, to determine $Fe^{\equiv}$, $Fe^{=}$ and organically bound iron in tissues and blood.

Some values recorded for the total inorganic iron content of whole blood, plasma and serum are:

Author	Inorganic iron content	
Hausermann [1899]	1·0 mg./100 cc.	calf-blood
Fontès and Thivolle [1925]	0·2 „	horse-serum
Heinriques and Roche [1927]	0·2 „	serum
Barkan [1927]	0·22 „	serum
„	1·34 „	whole blood
Starkenstein and Weder [1928]	1·9 „	dog-blood
„ „	1·1 „	protein-free plasma

Assuming the inorganic iron (ferrous and ferric) content of blood to be about 1·5 mg. per 100 cc. the 1 cc. sample taken by Pincussen and Roman to determine ferrous and ferric iron should contain about 0·015 mg. total inorganic iron. The iron equivalent of the titanous solution used by them was such that 1 cc. reduced 0·7 mg. $Fe^{\equiv}$. While one of the objects of the present paper is to

point out how extremely accurate and sensitive the titanium titration of iron is, we would not care to suggest its use to determine the $Fe^{=}$ and $Fe^{\equiv}$ content of a solution the total inorganic iron content of which is approximately 0·015 mg.

Where titanium titration has been employed to determine the iron content of organic substances, the end-point in the titration has heretofore been detected by potassium thiocyanate indicator. With hydrochloric acid solutions of ferric salts or of the ash of biological materials, this end-point is extremely sharp, and no advantage is to be gained by substituting any other method of end-point detection. We have found, however, that when titrating the ferric iron in a sulphuric-perchloric acid digest of an organic substance the indicator end-point is quite unsatisfactory.

In sulphuric acid solution the colour of the ferric thiocyanate fades slowly to a reddish-brown during the titration, and the exact end-point cannot be accurately detected[1]. Again there are occasions when it is desired to determine the iron content of solutions which are coloured. We have found that a much wider, and often more accurate, application of titanium titration can be made when the end-point is detected by measuring the change in potential of a suitable electrode during the titration.

Experimental

The following experiments are mainly concerned with the determination of iron in sulphuric-perchloric acid digests of organic materials by potentiometric titration with titanous sulphate.

I. *Apparatus.* A highly sensitive Leeds and Northrup "type P" galvanometer was used, and the difference in the potential of the platinum electrode was followed with a Leeds and Northrup "type K" potentiometer. The reference electrode was a saturated potassium chloride-calomel-platinum cell, which was connected to the titration vessel by a salt-bridge consisting of a capillary tube of 1 mm. inside diameter, filled with agar-agar potassium chloride gel. The electrode in the titration vessel was a $\frac{1}{2}$-inch spiral coil of bright platinum wire.

An accurately calibrated 10 cc. gas burette (Haldane gas analysis apparatus), the constricted 3 cc. portion of which was graduated in 1/100 cc. and a 500 cc. gas-washing bottle with ground-in stopper were used to construct the burette shown in Fig. 3. By means of this burette, the titanous sulphate solution is protected from air, does not come in contact with rubber during storage and can be accurately measured to 0·01 cc.

The titanous sulphate solution is poured into the bottle and the entire apparatus flushed with hydrogen through stopcocks A and B by adjusting stopcock C. The titanous sulphate solution is forced up into the burette by hydrogen pressure through A, with C adjusted and B open to the air. A and B are three-way stopcocks which are finally closed to the air but adjusted to give

[1] For a probable explanation, see Part I, The effect of acidity (p. 1035).

an open circuit within the burette. When stored in this manner the strength of the titanous sulphate solution undergoes relatively little change in two weeks. The solution should, however, be standardised on the day on which it is used.

The titration vessel has a capacity of 50 cc. and is arranged so that the titration can be carried out at 50° to 55° under carbon dioxide. The carbon dioxide is bubbled through a series of wash-bottles containing, in the following order, concentrated sulphuric acid, dilute titanous sulphate in sulphuric acid solution, pyrogallol and aqueous permanganate solution. The titration is best

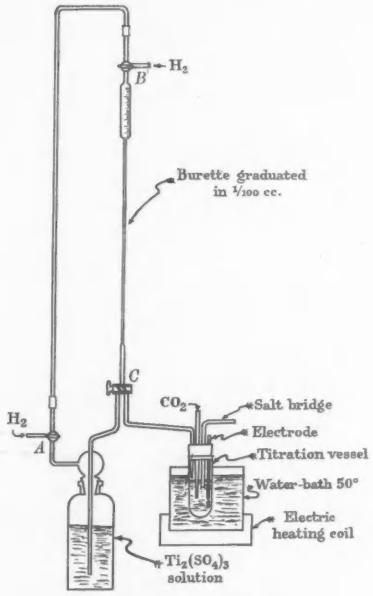

Fig. 3. Titration apparatus.

carried on at 50° to 60° as the last traces of Fe≡ are only slowly reduced at room temperatures.

II. *The titanous sulphate solution.* This solution was prepared by suitable dilution of a 20 % titanous sulphate solution (La Motte Chemical Co.) after adding the requisite amount of sulphuric acid to prevent hydrolysis with the formation of metatitanic acid. The solution was standardised against ferrous ammonium sulphate by the method recommended by Thornton and Chapman [1921] as follows.

A weighed portion of ferrous ammonium sulphate was dissolved in water acidified with 10 cc. 5 *N* sulphuric acid and titrated with *N*/100 permanganate solution, which was standardised against pure anhydrous sodium oxalate as the

primary standard. The titration was carried out with a micro-burette, and with carbon dioxide bubbling through the solution. At the completion of the oxidation the solution was boiled, cooled to 50°, 1 cc. 10 % potassium thiocyanate solution added, and titrated at 50° with the titanous sulphate solution. The end-points were detected by both the indicator and the voltage method. The titanous sulphate solution was also standardised in the same manner against a standard iron solution (1 cc. = 0·174 mg. Fe) prepared from "analytical" iron wire. This standard iron solution was used to determine the titre of the titanous solution each day it was used.

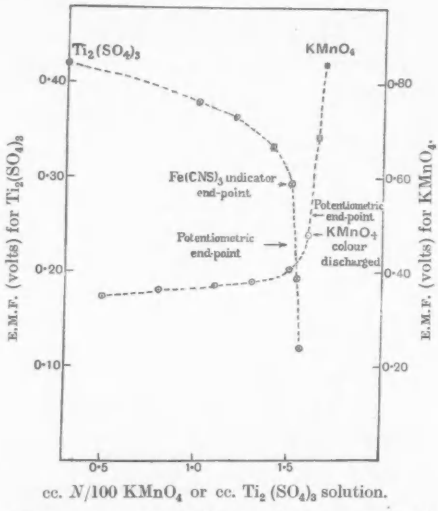

Fig. 4 Showing typical permanganate and titanium titration curves with 0·83 mg. iron.

To determine with what accuracy small amounts of iron could be estimated, and to get some idea of the sensitivity of the potentiometric method of detecting the end-point, a number of analyses of small quantities of ferrous ammonium sulphate, weighed on a micro-balance, were made.

Weight of ferrous ammonium sulphate mg.	Theoretical iron content mg.	Titanous sulphate titre in cc.	Iron value of 1 cc. titanous sulphate mg.
5·73	0·81	1·42	0·57
4·72	0·67	1·28	0·52
3·95	0·56	1·00	0·56
4·60	0·65	1·25	0·52
5·82	0·83	1·53	0·54

Typical titration curves with permanganate, and back titration with titanous sulphate, are reproduced in Fig. 4. In both titrations the change in potential in the vicinity of the end-point is always very large, enabling one to determine it without difficulty. The actual values of the potentials are highly

reproducible. The complete data of the titration curves are recorded for illustrative purposes only. It will be noted that in both titrations the indicator end-point just precedes the potentiometric end-point. In Fig. 5 are shown the results obtained on titrating, at 50°, 3 cc. of a standard iron solution (1 cc. = 0·174 mg. Fe) with the same titanous sulphate solution when 1 cc. 10 % potassium thiocyanate solution was added and the titration carried out in an atmosphere of carbon dioxide. The iron value of the titanous sulphate

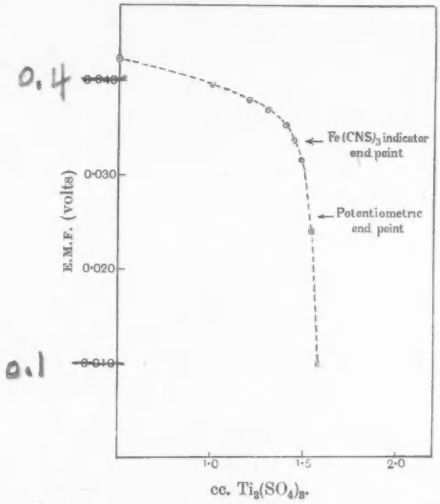

Fig. 5. Titanous sulphate titration of 0·522 mg. iron with 1 cc. 10 % potassium thiocyanate present.

solution was calculated from this titre. In this particular determination we have then:

Wt. of ferrous ammonium sulphate	5·82 mg.
Theoretical iron content, of ferrous ammonium sulphate	0·83 mg. Fe
1·62 cc. 0·00928 *N* $KMnO_4$ (Fig. 4)	0·84 ,,
1·52 cc. $Ti_2(SO_4)_3$ solution (Fig. 5)	0·80 ,,

According to Kolthoff and Furman [1926] the oxidation-reduction potential of titanic-titanous solutions depends to a large extent upon the p_H. The reducing action of titanous salts increases with increasing p_H. These workers have recommended titrating ferric iron in the presence of a large excess of Rochelle salt.

We have recorded in Fig. 6 the results of some of the experiments which we have made to determine the conditions for the maximum change in potential at the end-point. The first, second and third titration curves in each experiment are for 1, 2 and 3 cc. respectively of a standard iron solution (1 cc. = 0·174 mg. Fe). It will be seen that in experiment A (unbuffered) the end-point is ill-defined, while in experiments B and C the end-point is marked by an inflection

potential of at least 0·2 volts (against saturated calomel electrode). On repeating experiment B with widely varying amounts of Rochelle salt, the most satisfactory conditions were obtained when the solution was buffered with 0·1 g. Rochelle salt. 1 cc. of 10 % potassium thiocyanate (exp. C), while a very unsatisfactory indicator, was found to give the most favourable conditions for accurate end-point detection by electrometric means. Under the conditions of exp. C, each addition of the reducing agent is accompanied by a rapid establishment of a steady potential. At the completion of iron reduction, however, the E.M.F. drifts slowly (2 minutes) to zero. The inflection potential is not affected by pronounced changes in the sulphuric acid concentration.

Whilst potassium thiocyanate evidently acts as an excellent buffer in these titrations, it is difficult to explain the difference in the results obtained with potassium thiocyanate and Rochelle salt from the standpoint of buffer action

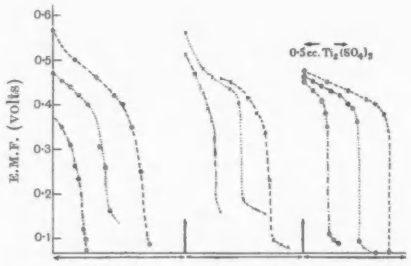

Fig. 6.

alone. The iron values of 1 cc. of the titanous sulphate solution, calculated from the potentiometric end-points (exp. C, Fig. 6) are 0·58, 0·57 and 0·57 mg. respectively.

In applying the voltage method of detecting the end-point to determinations of the iron content of sulphuric-perchloric acid digests of organic substances, we have added 1 cc. 10 % potassium thiocyanate before making the titration. An inflection potential of the same magnitude as that obtained in exp. C (Fig. 6) has always been obtained. As little as 0·10 mg. Fe≡ can thus be accurately determined. The change in colour of the ferric thiocyanate also affords a preliminary indication that the titration is nearing completion.

The iron content of milk.

In a previous paper the author [1932, 1] has described a method of estimating the copper content of whole milk (cows) in which the copper in a hydrochloric acid solution of the milk ash is precipitated as cupric sulphide. The iron content of the filtrate has been determined by titanium titration as follows.

The filtrate from the cupric sulphide precipitate was transferred to a 250 cc. pyrex Erlenmeyer flask and evaporated almost to dryness. A small filter-funnel was placed in the neck of the flask to prevent loss by bumping. To oxidise the iron, 2 cc. conc. nitric acid were added and the solution was boiled for a few minutes, cooled, 2 cc. conc. sulphuric acid added and the solution and calcium sulphate precipitate again boiled until the nitric acid and SO_3 fumes were completely evolved. Thorough boiling was necessary to decompose completely the nitrates of sodium and potassium, the presence of which interfere with the titanium titration. The solution was finally diluted with 10 cc. hot water and filtered into the titration vessel. The calcium sulphate precipitate was washed

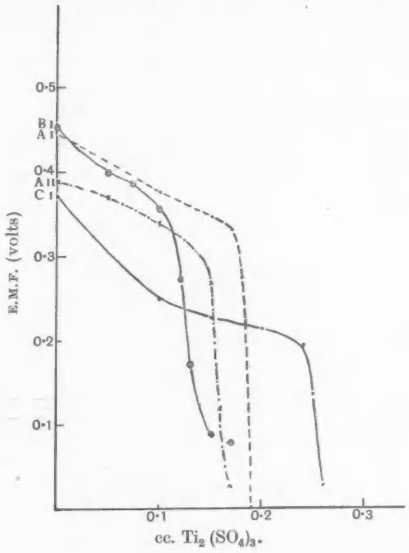

Fig. 7.

with two or three small portions of boiling water, and the washings were added to the filtrate. The potentiometric titration was made after adding 1 cc. 10 % potassium thiocyanate solution.

Complete decomposition of the alkali nitrates may be more thoroughly assured by evaporating the filtrate from the calcium sulphate precipitate almost to dryness in a beaker covered by a watch glass, taking up with water and carrying out the titration.

A steady "initial" potential should be obtained after bubbling carbon dioxide through the solution for a few minutes. If alkali nitrates are still present, side-reactions take place and a drifting potential results. Until the end-point is reached, a steady potential is almost instantaneously obtained after each addition of titanous sulphate. At the end-point when the E.M.F. is about 0·35 v.

the potential slowly (2 minutes) drifts to zero. In such titrations the indicator end-point is extremely indistinct.

The iron contents of a number of samples of milk have been determined by the above procedure. The titration curves are shown in Fig. 7. The results may be summarised as follows:

	Sample of milk	Amount of milk analysed cc.	$Ti_2(SO_4)_3$ (voltage end-point) cc.	Iron content of milk mg./litre
A	1	250	0·18	0·53
	2	150	0·15	0·57
	3*	150	0·14	0·55
B	1	100	0·12	0·68
	2*	150	0·16	0·64
C	1	300	0·25	0·48

* Results not plotted in Fig. 7. *Note*: 1 cc. $Ti_2(SO_4)_3$ solution reduced 0·57 mg. Fe.

The results of duplicate and triplicate analyses are in good agreement. The most recent iron determinations on milk are those recorded by Stugart [1931] using a modification of Kennedy's method. He records values from 0·44 to 0·53 mg. iron per kg.

Iron content of lecitho-vitellin.

The iron content of two samples of lecitho-vitellin [for method of preparation, *etc.* see McFarlane, 1932, 2] was determined. The samples were digested with sulphuric-perchloric acid as described in Part I of this paper[1]. The digest was diluted with water, transferred to the titration vessel and, after adding 1 cc. 10 % potassium thiocyanate solution, titrated with titanous sulphate. The end-point was determined electrometrically.

Sample of lecitho-vitellin		% iron		Iron titrated mg.
No.	Weight g.	Titanium titration	Micro-colorimetry (See Part I)	
1.	0·3050	0·047	0·045	0·14
2.	0·4272	0·043	0·042	0·18

Iron content of crystalline haemoglobin.

The source of this particular preparation was unknown. The sample was digested as described for lecitho-vitellin. The "initial" potential before adding titanous sulphate was extremely unsteady. This difficulty was overcome by adding an excess of bromine water (1 to 2 cc.) at the completion of digestion. The digest was boiled until the excess bromine was driven off. It was then cooled, and titrated as before with 1 cc. 10 % potassium thiocyanate solution present.

[1] 2 cc. concentrated sulphuric acid and 1 cc. 60 % perchloric acid were, however, required for the digestion.

Wt. of crystalline haemoglobin g.	% iron	Iron titrated mg.
0·0903	0·328	0·29
0·1907	0·316	0·59
0·0427	0·322	0·14

Iron content of blood.

The iron content of oxalated human blood was determined by the same procedure as that described for crystalline haemoglobin. The final oxidation with bromine, already mentioned, greatly improved the results. The following values illustrate the accuracy of the method:

Amount of blood analysed cc.	% Iron	Iron titrated mg.
2·0	0·052	1·05
1·0	0·051	0·51
0·5	0·051	0·26

The haemoglobin content of this sample of blood was 14·62 % as determined by oxygen capacity [Sendroy, 1931]. Assuming that 1 g. haemoglobin contains approximately 0·0032 g. iron (see above), the iron content should be about 0·048 %. A determination of the iron content of blood can be made in less than 30 minutes.

Iron content of liver.

According to Starkenstein and Weder [1928], the inorganic iron, and none of the iron in organic combination, is extracted from blood or tissue by boiling with 5 *N* hydrochloric acid. The protein-free filtrate (trichloroacetic acid) contains the whole of the inorganic iron. We have found by applying these and our own findings already recorded in this paper that the inorganic and organic iron contents of liver tissue can be determined rapidly and accurately.

A weighed portion of rat-liver, about 1 g. is pulverised with a small amount of quartz sand[1] in a mortar, and then transferred to a 100 cc. pyrex centrifuge-tube with the aid of 10 cc. 5 *N* hydrochloric acid[2]. After boiling about 10 minutes the solution is cooled and the protein precipitated with 20 % trichloroacetic acid. After centrifuging, the clear supernatant liquid is transferred to an Erlenmeyer flask and evaporated to a small volume. The iron is completely oxidised with saturated bromine water, and the excess bromine boiled off. The inorganic iron is determined as ferric iron by titrating with titanous sulphate, as already described. The protein residue in the centrifuge-tube is oxidised with sulphuric-perchloric acid and the "organic" iron determined by the procedure recommended for blood. The accuracy of the method is comparable with that for blood. The inorganic iron content of adult rat-liver (calculated on the fresh weight) was found to vary from 0·017 to 0·058 % and the "organic" iron from 0·72 to 1·13 %. Detailed observations in this regard will be recorded in a later paper.

[1] The quartz sand was freed from iron by extraction with hot concentrated hydrochloric acid.

[2] The hydrochloric acid must be freed from iron by redistilling.

SUMMARY.

Part I.

The method of Kennedy [1927] for the estimation of iron in organic materials has been studied with a view to applying this procedure to the micro-colorimetric estimation of minute amounts of iron in organic substances. Under the conditions described, and with the quantities of iron being determined (0·007 to 0·022 mg.) the intensity of the red colour of ferric thiocyanate is not proportional to the concentration of iron present. It is possible to correct for this deviation from Beer's Law so that the iron content of as little as 0·03 g. of lecitho-vitellin (0·014 mg. Fe) can be accurately estimated.

Part II.

Methods are described for the rapid and accurate estimation of as little as 0·15 mg. of iron in a sulphuric-perchloric acid digest of an organic substance by potentiometric titration with titanous sulphate. A modification of the procedure is outlined, whereby the total iron contents of milk and blood, and the total "inorganic" and "organic" iron contents of tissues may be determined.

The author desires to express his indebtedness to Dr O. J. Walker, of the Department of Chemistry, for helpful advice and to Prof. George Hunter for his interest in this work and his valued criticism of the manuscript. He is also indebted to Mr Wardlow Porteous for his drawing of the titration apparatus.

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CXXIII. ON THE FACTORS INFLUENCING THE BLOOD-BENZIDINE REACTION AS APPLIED TO THE MICRO-DETERMINATION OF HAEMOGLOBIN.

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We have been surprised to observe that, notwithstanding the increasing need for an accurate method of determining minute amounts of haemoglobin, the method recommended by Wu [1923] has not been more generally adopted. This author developed from the benzidine test for blood a quantitative "ultra-micro-method" by means of which the haemoglobin content of 0·0001 cc. of blood could be accurately determined.

The accuracy of the method proposed by Wu has recently been questioned by Bing and Baker [1931], who have proposed a modified procedure which, they claim, eliminates certain errors in the original method. We have recorded in this paper a critical study of the blood-benzidine reaction, particularly as affected by hydrogen peroxide concentration and temperature. Other observations having a direct bearing on the quantitative application of the reaction are discussed.

Experimental.

The chemical reactivity of benzidine, particularly so far as oxidation is concerned, depends on its purity and on the method of preparation. To interpret our experimental results it was essential that benzidine of known purity should be employed.

Purification of benzidine. To 100 g. finely powdered commercial benzidine in a two-litre flask were added 900 cc. boiling water and 100 cc. conc. hydrochloric acid. After boiling a short time the hot solution was filtered, using suction. The filtrate was again heated to boiling and 230 cc. conc. hydrochloric acid, in 30–50 cc. portions, were added, shaking after each addition until the grey precipitate dissolved. A permanent precipitate formed when about 150 to 175 cc. had been added. After filtering (suction) the highly coloured filtrate was discarded and the precipitate transferred to a two-litre flask. One litre of boiling water and a tablespoonful of blood-charcoal (acid-extracted) were added, and after boiling about 10 minutes the hot solution was again filtered (suction). This procedure was repeated until the filtrate was colourless. To the hot filtrate 230 cc. conc. hydrochloric acid in 30 to 50 cc. portions were added while shaking, and after cooling to 20° again filtered.

[1] Robert Tegler Research Scholar.

To obtain pure benzidine, 100 g. of the hydrochloride were suspended in 600 cc. acetone, 100 cc. conc. ammonium hydroxide and 150 cc. of water. After the benzidine was completely dissolved, the solution was diluted to 5 litres with water and filtered after standing 20 minutes. The benzidine was washed free from chloride with water, and finally dried over sulphuric acid in a vacuum desiccator. The benzidine thus prepared was found to be extremely insoluble in glacial acetic acid. This difficulty was overcome by recrystallising from hot 95 % ethyl alcohol.

The benzidine solution used in all our experiments was prepared by dissolving 2 g. of the pure benzidine in 20 cc. of glacial acetic acid with the aid of heat, and, after cooling, diluting to 100 cc.

Hydrogen peroxide. All solutions of hydrogen peroxide were prepared by suitable dilution of "Superoxol." The hydrogen peroxide contents of these solutions were carefully checked by permanganate titration, using pure anhydrous sodium oxalate as the primary standard. It was not considered advisable, at least so far as our experiments were concerned, to use the commercial 3 % H_2O_2 solution, which is generally stabilised by acetanilide or by small amounts of sulphuric acid. Hydrogen peroxide forms compounds with sulphuric acid in which the peroxide is less easily decomposable than when alone.

Standard blood solution. The haemoglobin content of a sample of sheep's blood was estimated from its oxygen capacity, as determined by Sendroy's [1931] modification of the Van Slyke and Neill [1924] procedure. This blood was diluted to contain 0·05 mg. haemoglobin in 1 cc. Boric acid was used as the preservative.

General experimental conditions. Throughout the experiments, special precautions were taken in regard to the cleanliness of all glassware [see Harrison, 1930]. In carrying out the benzidine reaction the reagents were always added in the order recommended by Wu. To 2 cc. of a 2 % benzidine solution is added 1 cc. diluted blood. After carefully mixing, 1 cc. of a hydrogen peroxide solution is added. After a definite reaction time, the solution is diluted with 21 cc. 20 % acetic acid, and the intensity of the reddish-brown colour measured in a colorimeter of the Duboscq type.

The effect of the concentration of hydrogen peroxide on the benzidine reaction and on the stability of the red colour.

The essential feature of the modified procedure recommended by Bing and Baker is the substitution of a much more dilute (0·6 %) H_2O_2 solution for the 3 % H_2O_2 solution used by Wu. This permits an extension of the reaction time of 15 minutes, as specified by Wu, to one of 1 hour or longer, which is an undoubted convenience when many determinations are being made. It is particularly unfortunate that the experimental data justifying the use of more dilute H_2O_2 are not given.

Our results on the effect of hydrogen peroxide concentration on the development and on the stability of the red colour are given in Table I. The

Table I. *The effect of H_2O_2 concentrations on the benzidine reaction.*

Reaction time, 1 hour. Temperature, 20–22°.

Solution No.	Percentage concentration of H_2O_2	Reading of the standard (No. 4) in mm. with the other test solutions set at 10 mm.	
		After 15 minutes	After 22 hours
1	4·0	11·8	9·8
2	3·0	11·1	9·8
3	2·0	9·9	10·3
4	1·5	10·0	10·0
5	1·0	8·4	10·3
6	0·75	7·6	10·2
7	0·50	6·3	8·8
8	0·25	5·1	7·9

data were obtained as follows. The benzidine reaction was carried out with 1 cc. of hydrogen peroxide solutions of varying strength. The reaction was allowed to proceed exactly 1 hour (in all cases) when the solutions were diluted with 21 cc. 20 % acetic acid. After 15 minutes the comparative depth of colour was measured, with solution No. 4 (1·5 % H_2O_2) as the arbitrary standard. The test solutions were always set at 10 mm. and the standard adjusted to match. In this way the numerical value of the readings is shown as increasing with increasing intensity of colour. Using a new standard prepared exactly as solution No. 4, the relative stability of the colours was determined after 22 hours. The two standards when read against each other had practically the same depth of colour.

We can conclude that, as the concentration of hydrogen peroxide is increased from 0·25 % to 4 %, the intensity of the red colour also continues to increase. The maximum intensity of the colour consistent with the greatest stability was obtained with 1·5 % or 2 % of H_2O_2. 0·75 % H_2O_2 gave only 76 % of the colour given by 1·5 % H_2O_2.

Table II. *Effect of H_2O_2 concentration on the development and stability of the red colour.*

Reaction time, 1 hour. Temperature, 20–22°.

Solution No.	Percentage concentration H_2O_2	Reading of standard in mm. with other test solutions set at 10 mm.			
		*15 minutes	2 hours	3 hours	3 hours
1	10	11·8	10·5	8·8	7·9
2	8	12·0	11·2	9·7	8·7
3	6	11·6	10·2	9·4	8·5
4	4	11·2	11·0	10·5	10·0
5	2	10·3	10·2	9·9	9·9
6	1·5	10·0	10·3	10·2	10·2
7	1·0	8·4	8·9	9·8	10·2
8	0·5	6·8	7·4	8·4	8·9

Tube No. 6 (1·5 % H_2O_2) was used as standard in the first series of readings, *i.e.* after 15 minutes. A fresh standard prepared exactly as No. 6 was used for each series of readings.

* Time after dilution with 20 % acetic acid.

In Table II we have recorded the results obtained when a wider range of H_2O_2 concentrations was employed. The effect of the peroxide concentration on the stability of the red colour was determined hourly up to 4 hours. The same experimental conditions prevailed as those described above. Again 1·5 % H_2O_2 was the lowest concentration which gave the maximum depth of colour, stable for at least 4 hours. Hydrogen peroxide concentrations of 4 % or greater gave more colour, but the colour was less stable, the instability increasing with increasing H_2O_2 concentration. The intensity of the colour given by 1·0 % and 0·5 % H_2O_2, which is initially much less than that given by 1·5 % H_2O_2, slowly increased when the solutions were allowed to stand 4 hours.

The results of a further experiment similar to those already described, but in which the reaction time was varied, are given in Table III. By "reaction

Table III. *Effect of varying concentrations of H_2O_2 on the development and stability of the red colour.*

Reaction time varying, room temperatures prevailing.
Reading of standard in mm. with other test solutions set at 10 mm.

Solution No.	Reaction time	3 % H_2O_2				1·5 % H_2O_2				0·5 % H_2O_2	
		15 mins.	2 hrs.	3 hrs.	15 hrs.	15 mins.	2 hrs.	3 hrs.	15 hrs.	15 mins.	15 hrs.
1	2 hrs.	cloudy	—	—	—	13·1	12·7	12·8	11·9	9·5	10·6
2	1·5 „	cloudy	—	—	—	11·2	10·9	11·0	9·8	8·7	10·1
3	1·0 hr.	12·6	12·0	11·6	10·3	10·0	10·3	10·3	9·8	8·1	10·0
4	45 mins.	11·3	11·0	11·1	9·3	10·1	10·0	10·2	10·0	7·6	9·3
5	30 „	10·8	10·7	10·9	9·5	9·6	10·1	10·3	9·8	6·7	9·0
6	15 „	10·2	10·0	10·0	8·4	8·1	8·9	9·1	9·0	5·2	8·8

Note. A freshly prepared standard was used for each series of readings after 15 minutes, 2 hours, *etc.* The preparation of the standard was always the same, *i.e.* using 1·5 % H_2O_2, reaction time, 1 hour, and allowing the solution to stand 15 minutes after dilution with 20 % acetic acid.

time" is meant the length of time the benzidine reaction is allowed to proceed before dilution to 25 cc. with 20 % acetic acid. The conclusion may be drawn from these results that 1·5 % H_2O_2 and a reaction time of 45–60 minutes give the maximum depth of colour, the intensity of which undergoes little or no change on standing 15 hours. The stability of the colour with 3 % H_2O_2 when the reaction time is 15–45 minutes is noteworthy.

Consideration of the data obtained from these experiments suggests that the benzidine reaction may be carried out with the greatest sensitivity, accuracy and convenience, when 1·5 % H_2O_2 with a reaction time of 1 hour is employed.

The effect of temperature on the colour development.

To study the effect of temperature, the following procedure was adopted. The benzidine reactions were carried out in 25 cc. glass-stoppered measuring cylinders. These were placed in a constant temperature water-bath. From micro-burettes were added 2 cc. 2 % benzidine solution and 1 cc. diluted blood (0·05 mg. haemoglobin) and the solutions thoroughly mixed. They were then allowed to stand until the reagents came to the temperature of the water-bath (about 3 minutes). 1 cc. of 1·5 % H_2O_2 was then added. At the end of the particular reaction time the cylinder was removed from the water-bath and 21 cc. of cold (about 2°) 20 % acetic acid were quickly added. The colorimeter readings were made 15 minutes later.

The benzidine reaction was also carried out in the same manner at 2·2° (refrigerator room).

The temperature at which the benzidine reaction is performed has evidently a marked effect upon the rate of the "initial" stage of the reaction through the blue colour to the red (Table IV). When the reaction was carried out at 2·2° the solution was still deep blue in colour after a reaction time of 1 hour. At 50° a reaction time of 2·5 minutes gave the same depth of colour as that obtained in 15 minutes at room temperatures. It appears that after the red

Table IV. *Effect of temperature on the benzidine reaction.*

Reaction time minutes	Reading of standard with other test solutions set at 10 mm.				
	20–22°	30°	40°	50°	60°
2·5	*—	—	—	7·0	—
3	—	—	—	7·6	—
5	—	5·0	7·0	8·0	7·2
10	—	6·7	—	8·2	7·4
15	7·1	7·1	8·2	8·6	7·5
20	8·3	7·8	—	8·6	—
25	8·8	8·5	—	9·4	—
30	9·2	8·5	9·2	9·4	7·5
45	10·2	9·5	9·7	9·6	Cloudy
60	10·0	10·0	9·9	9·2	—

Note. These experiments were completed on the same day, so that the same standard, using 1·5 % H_2O_2 and a 1-hour reaction time at room temperatures, were used for all readings. 1·5 % H_2O_2 was used in all the tests.

* The reaction had not proceeded to the red colour. The solution after dilution with 20 % acetic acid had a greenish-red colour.

colour has developed variations in temperature from 20° to at least 40° have very little effect on the rate at which this colour reaches its maximum intensity. The depth of colour developed after 60 minutes, using 1·5 % H_2O_2, is the same whether the reaction is carried out at 20° or at 40°.

The results of a further experiment in which the reaction was carried out with three different strengths of hydrogen peroxide, and in which the temperature was carefully controlled, are shown graphically in Fig. 1. When the

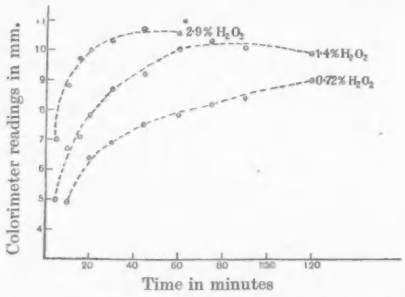

Fig. 1. Showing the effect of varying concentrations of H_2O_2 on the rate of substrate (benzidine) oxidation.
* Solutions became cloudy.

temperature is constant at 30°, increasing the concentration of H_2O_2 not only accelerates the initial stage of the reaction, but the time required for the red colour to reach an intensity equal to that of the standard is markedly shortened.

The effect of light on the colour development.

When the benzidine reaction, using 1·5 % H_2O_2, was carried out in the dark and in the light we found no difference in the depth of colour developed after a reaction time of 1 hour.

A few experiments were carried out to determine whether the rate of the reaction was influenced by radiations in the ultra-violet. It was only possible to draw very general conclusions from these results, because of the difficulty in controlling other experimental conditions. Bernheim and Dixon [1928] have found in studying the effect of light on the reduction of methylene blue by the xanthine oxidase system with oxygen present, that a peroxide is formed during the irradiation. There was no doubt, however, that exposure of the reaction mixture to the radiations of a quartz mercury vapour lamp, set 12″ directly above the solution, greatly increased the rate of the reaction. An exposure of 6 to 7 minutes gave about the same depth of colour as ordinarily develops in one hour in daylight. A cloudiness developed in the reaction mixture if irradiated for more than 7 minutes.

The effect of varying haemoglobin concentrations on the intensity of the colour.

Varying amounts from 2 cc. to 20 cc. of a dilute blood solution containing 13·5 mg. of haemoglobin in 100 cc. were measured into 25 cc. volumetric flasks. After diluting to volume, the benzidine reaction (2 cc. benzidine solution; 1 cc. 1·5 % H_2O_2; room temperature and reaction time 1 hour) was carried out with 1 cc. aliquots of the different blood dilutions. 21 cc. of 20 % acetic acid were then added and the solution allowed to stand 10 minutes before making the colorimeter readings. The range of haemoglobin concentration was from 0·01 mg. to 0·10 mg. per cc. The fifth dilution (10 cc. of diluted blood in 25 cc.) was taken as the arbitrary standard.

In Fig. 2 are plotted the colorimeter readings of this standard when ad-

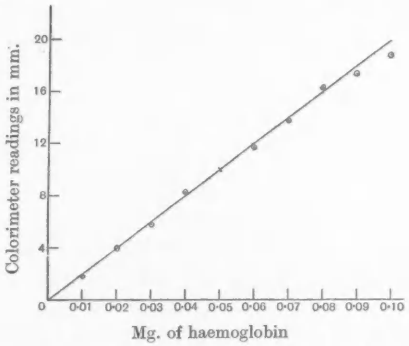

Fig. 2. Showing the relationship between the haemoglobin concentration and the intensity of the red colour.

justed to match each of the other test dilutions which were set at 10 mm. The depth of colour is directly proportional to the amount of haemoglobin present, provided the standard selected is used over a limited range of haemoglobin concentrations.

The determination of haemoglobin in small amounts of blood.

For some considerable time we have used Wu's benzidine method for the routine estimation of haemoglobin in minute amounts of rat-blood. As a result of the experimental findings already discussed, certain changes have been made in the original procedure. These modifications constitute an improvement only so far as technique is concerned.

The accuracy with which the haemoglobin content of rat-blood can be repeatedly determined is limited primarily by the error incurred in measuring small samples of blood from a free-flowing drop taken from the tail. We have used a red cell counting pipette which was accurately calibrated and found to deliver 0·00245 cc. blood from the 0·05 mark. The accuracy with which samples of blood could be procured by means of this pipette was determined as follows.

The tail was wiped clean (if the rat was anaemic the tail was immersed for a time in warm water) and the end clipped off. The sample was pipetted from the second or third drop of blood, and transferred to a 10 cc. volumetric flask containing a small amount of water with which to flush the pipette. The blood solution was diluted to 10 cc., thoroughly mixed, and the benzidine reaction carried out in the same manner as in conducting the proportionality tests. A number of samples were taken from the same and from different rats. The final colorimeter readings were all made against the same standard (0·028 mg. haemoglobin) which was adjusted to match the test solution set at 10 mm.

In all the experiments described, specially purified benzidine has been used. Quantitative determinations of equal accuracy may be made, using Merck's benzidine "suitable for detecting blood."

	Sample of blood	Colorimeter reading		Sample of blood	Colorimeter reading
Rat No. 1	1	12·0	Rat No. 2	1	9·8
	2	11·5		2	9·4
	3	11·5		3	9·4
	4	11·7		4	10·0
Rat No. 3	1	9·8	Rat No. 4	1	13·0
	2	9·8		2	12·8
	3	10·2	Rat No. 5	1	12·7
	4	10·0		2	12·2

The greatest variation in these colorimeter readings is 0·6 mm., which may be attributed almost entirely to inaccuracies in the measurement of small samples of blood by the method described. This error is equivalent to an error of 0·7 % haemoglobin in the original sample of blood.

So far we have been primarily concerned with the empirical standardisation of the benzidine reaction, so that it might be applied to comparative haemoglobin determinations in rat's blood. Haemoglobin estimations on the same sample of oxalated blood have been made by the colorimetric method and by oxygen capacity determinations. Since the colorimetric method is based on the oxygen capacity determinations, a comparison of the results obtained by the two methods is of no significance, so far as the absolute accuracy of the colorimetric method is concerned. However, since new standards have to be prepared from time to time, the relation of one series of colorimetric determinations to another depends upon the accuracy with which the oxygen capacity determinations check with the colorimetric method.

		g. haemoglobin in 100 cc. blood		
	Sample of blood	Colorimetric method	Calc. from O_2 capacity [Sendroy, 1931]	
Human blood	*1	14·3		
	2	13·8	13·93	Average
	3	13·7	14·23	14·08
	4	13·7		
Sheep-blood	1	13·9		
	2	13·6	13·97	
	3	14·2		
Rat-blood No. 3.	1	14·0	14·4	
	2	14·0		
Rat-blood No. 4	1	10·1	9·64	
	2	9·9		

* Aliquots of the same sample of blood.

The effect of heating the diluted blood.

Three 10 cc. aliquots of a diluted blood (13·5 mg. haemoglobin in 100 cc.) were measured into 25 cc. volumetric flasks. Two of the flasks were placed in a constant temperature water-bath at 60°. One was removed at the end of 30 minutes and cooled to room temperature, while the other remained in the water-bath for 1 hour. The contents were all diluted to 25 cc., then thoroughly mixed and the benzidine reaction was carried out on a 1 cc. aliquot (0·054 mg. haemoglobin).

No.	Treatment of diluted blood	Colorimeter readings in mm. of No. 1 as standard with other test solutions set at 10 mm.
1	Unheated	10·0
2	Heated for 30 mins. at 60°	9·1
3	Heated for 1 hour at 60°	9·0

These observations have been repeated independently by both authors with different samples of human, sheep- and rat-blood. About 10 % of the peroxidase activity is destroyed by heating the diluted blood at 60° for 30 minutes. The activity is unchanged when the sample is held at the same temperature for 1 hour.

General discussion.

To those familiar with the blood-benzidine reaction, it is evident that the quantitative application is only possible when the use of each reagent has been standardised. In this way only can those conditions be maintained which are necessary to preserve a constant peroxidase activity of the blood. The amount of benzidine transformed into the red oxidation product in a given time should then be directly proportional to the quantity of peroxidase (haemoglobin) present.

The greatest difficulty encountered in controlling the reaction, so that it may form the basis of a colorimetric procedure which is free from objections, is to

establish the optimum concentration of hydrogen peroxide. According to Wu, when 3 % H_2O_2 is used, the peroxidase reaction reaches a maximum in about 25 minutes, the "haemoglobin being all destroyed by the peroxide." The depth of colour, after 25 minutes, slowly increased, "due to direct oxidation of the benzidine by the peroxide alone." No explanation is offered as to how the cessation of peroxide activity (indirect oxidation) was differentiated from the commencement of the direct oxidation of the benzidine by the hydrogen peroxide. Even more indefinite and confusing is the statement of Bing and Baker that "We feel these better results (using 0·6 % H_2O_2) are due in part to a lessened tendency for the destruction of the haemoglobin activity by stronger peroxide solutions, and in part to the avoidance of the direct oxidation of the benzidine."

It is at once obvious that if these suggestions are tenable they preclude any possibility of developing an accurate quantitative colorimetric method based on the benzidine blood reaction. We can find no evidence to support the supposition that the benzidine may be directly oxidised by the excess hydrogen peroxide, or that the peroxidase activity of oxyhaemoglobin may be destroyed by stronger (presumably 3 %) peroxide solutions when benzidine is also present. The destruction of the peroxidase activity of dilute blood by hydrogen peroxide in the absence of benzidine is no evidence that the same reaction may take place in the presence of a readily oxidised substance. On the contrary, Willstätter and Stoll [1918] have shown that the catalytic function of oxyhaemoglobin, in contrast with the true plant peroxidase, increases pronouncedly with rising hydrogen peroxide concentration. Oxyhaemoglobin acts as a peroxidase without accelerating the destruction of hydrogen peroxide to any detectable extent [see Kuhn and Brann, 1926]. Again, our own observations on the increase in colour intensity with increasing concentrations of hydrogen peroxide from 0·5 % to 10 % (see Table II), while difficult to interpret, certainly do not support the above contentions. For the sake of clarity the first series of readings recorded in Table II are plotted in Fig. 3.

A mixture of 2 cc. of our benzidine solution and 1 cc. 3 % H_2O_2, when allowed to stand at room temperatures for 2 hours, developed only a faint yellow tinge. There is no reason to suspect that direct oxidation of the benzidine by 3 % H_2O_2 is any more likely to occur when the dilute blood is present. The values plotted in Fig. 3 represent the amounts of substrate (benzidine) oxidised by varying amounts of H_2O_2 in a given time (1 hour) with a constant quantity of the catalyst present. The amount of substrate oxidised in 1 hour increases with increasing H_2O_2 concentrations, and reaches a maximum with 8 % H_2O_2.

To form the basis of an accurate colorimetric method, the optimum concentration of H_2O_2 is considered to be that which gives the maximum depth of colour consistent with greatest stability. Under the conditions of our experiments this was found to be 1·5 % hydrogen peroxide.

The literature contains ample evidence to support the conclusions that the leucocytes contain a true (thermolabile) peroxidase. At such large dilutions

as those employed above, one would conclude the true peroxidase activity to be quantitatively negligible compared with the pseudo-peroxidase (thermostable) activity of oxyhaemoglobin. 10 % of the peroxidase activity of diluted blood is destroyed by heating at 60° for 30 minutes. Whether this is due to the destruction of a thermolabile peroxidase or to some change in the oxyhaemoglobin molecule remains to be determined. Kuhn and Brann [1926] have

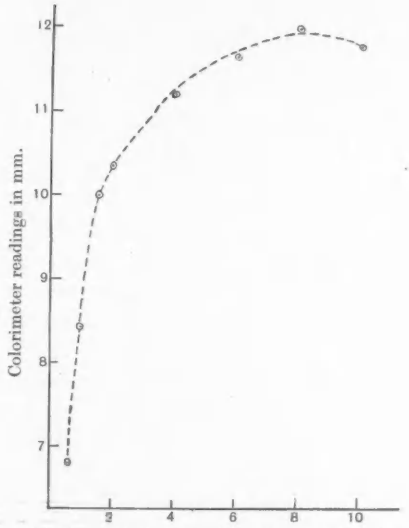

Fig. 3.

shown that relatively unimportant chemical alterations in the haemoglobin molecule markedly change the peroxidase activity. When the opportunity presents itself we plan to repeat our observation on the effect of heating the diluted blood, using pathological bloods with very high leucocyte contents, such as occur in leucaemias. Delhougne [1929] has reported that the true peroxidase number of blood as determined by the method of Bach and Zubkowa [1921] is sharply increased by vigorous exercise and in pathological conditions.

Summary.

1. Certain factors influencing the blood-benzidine reaction have been studied, with the following results.

(*a*) The amount of benzidine oxidised in 1 hour by hydrogen peroxide in the presence of a fixed amount of the catalyst (haemoglobin) increases with increasing hydrogen peroxide concentration. A maximum is reached with an 8 % solution of hydrogen peroxide. The maximum amount of the red oxidation

product which is stable for at least 12 hours, under the conditions of the blood-benzidine reaction, is given by a 1·5 % solution of hydrogen peroxide.

(*b*) The initial reaction, through the blue colour to the red, is markedly accelerated by increasing temperature. The rate at which the red colour reaches its maximum intensity is not influenced by variations in temperature from 20° to 40°.

(*c*) Light has no effect on the colour development. Radiations from a quartz mercury vapour lamp greatly increase the rate of the reaction.

(*d*) Heating diluted blood at 60° for 30 minutes destroys about 10 % of its peroxidase activity. The inactivation is not increased by heating for 1 hour at 60°.

(*e*) When the reaction is carried out with a 1·5 % solution of hydrogen peroxide and a reaction time of 1 hour at room temperatures, the peroxidase activity within the range of dilutions studied, is proportional to the concentration of haemoglobin.

2. The benzidine reaction, modified in accordance with the results summarised above, has been applied to the estimation of haemoglobin in minute amounts of blood. The accuracy of these estimations depends primarily upon the accuracy with which small samples (*e.g.* 0·0025 cc.) can be measured.

The authors wish to express their sincere gratitude to Professor George Hunter for his advice and valuable criticism.

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CXXIV. THE ALKALINE HYDROLYSIS OF LECITHO-VITELLIN WITH PARTICULAR REFERENCE TO THE SEPARATION OF AN ORGANIC FRACTION CONTAINING IRON AND COPPER.

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Miescher [1870] observed that after prolonged peptic digestion of egg-yolk an insoluble substance separated out. This substance was found by Bunge [1885] to contain iron in organic combination and was named by him "haematogen" because of its close resemblance to haemoglobin, at least so far as empirical composition was concerned. The chemical nature of this substance was investigated in more detail by Hugounenq and Morel [1905, 1, 2; 1906]. Again, Posternak [1927] has studied a similar iron-containing enzyme-resisting substance. The inconsistent results obtained for the iron content of this material indicate that haematogen is not a pure compound. Haematogen was regarded by these workers as a prosthetic group in the vitellin molecule and as being a precursor of the haemoglobin formed during avian embryonic development. A more detailed discussion of their findings is given by McFarlane, Fulmer and Jukes [1930] and by Jukes and Kay [1932].

Levene and Alsberg [1900] have obtained from vitellin an iron-containing substance, vitellinic acid, by the action of strong ammonium hydroxide. It is probable that vitellinic acid is a purer substance than haematogen since these workers avoided enzymic hydrolysis and employed the protein, vitellin, instead of egg-yolk. All these investigations were, however, carried out without knowledge of the presence of a second protein in egg-yolk. Livetin, this second protein [Plimmer, 1908], was first prepared by Kay and Marshall [1928] in pure form.

The constitution of the egg-yolk-protein has long been considered to resemble that of the milk-proteins. Rimington and Kay [1926] found that very mild hydrolysis of caseinogen with 1 % sodium hydroxide liberates the organic phosphorus from the protein molecule as inorganic phosphorus. We were therefore interested in determining what action dilute sodium hydroxide would have on lecitho-vitellin. In the course of this investigation we obtained results which indicated that the lecitho-vitellin molecule contained copper as well as

iron. These two elements were found in organic combination in the same fraction of a lecitho-vitellin hydrolysate.

Preparation of lecitho-vitellin from egg-yolk.

In endeavouring to improve upon existing methods of preparing the pure protein we have benefited considerably from the observations made by Kay and Marshall in the course of their preparation of livetin.

The intact yolks were freed from egg-white by washing first in running water and then under a slow stream of 0·9 % NaCl on filter-paper. The vitelline membrane was punctured so that the yolk contents were transferred to a beaker without the membrane and chalazae. The yolks, diluted with an equal volume of 10 % NaCl, were repeatedly extracted with ether until the ether extract was colourless. This was carried out, using a mechanical shaker, in a refrigerator room at −9·4° and required five twelve-hour extractions, each with an amount of ether equal to the volume of the egg-yolk solution. The saline solution was dialysed in a large parchment dialyser for two days at about −1·1° against repeated changes of distilled water. A large precipitate of the globulin formed. To complete the precipitation the solution was diluted with four volumes of ice-cold water and after standing overnight was rapidly centrifuged. The protein was dissolved in the minimum amount of 10 % NaCl, transferred to the dialyser, and the precipitation and separation repeated. A third solution and precipitation were carried out with the loss of a small amount of the protein which failed to dissolve in 10 % NaCl. The protein precipitate was transferred to an extraction bottle and extracted with alcohol-ether (seven parts alcohol and three parts ether) at −9·4° The extraction was repeated until no visible residue was obtained when 50 cc. of the extract were evaporated to dryness. The lecitho-vitellin was next extracted for 24 hours with anhydrous ether in a large glass Soxhlet apparatus with metallic sodium in the extraction flask [Hewitt, 1927]. The lecitho-vitellin was ground to a fine white powder and dried in a vacuum desiccator over sulphuric acid.

A very small portion of the protein thus prepared was insoluble in 0·1 *N* sodium hydroxide. On applying the nitroprusside reaction we found that these suspended particles developed on their surface a strong purple colour indicative of denaturation [Hopkins, 1930]. The soluble protein did not react. The following analyses of the lecitho-vitellin have been made:

	%
Nitrogen	15·71
Phosphorus [Richards and Godden, 1924]	0·59
Lecithin	2·17
Iron [McFarlane, 1932, 2]	0·047

Note. 1 g. of lecitho-vitellin was boiled with absolute alcohol for 24 hours in an atmosphere of an inert gas. The material thus extracted is believed to be lecithin. The dried residue, vitellin, is light reddish-brown in colour. When the extraction is made in the presence of oxygen the vitellin has a pink colour.

The action of 0·25 N sodium hydroxide on lecitho-vitellin.

To 2·0316 g. lecitho-vitellin in a 750 cc. Erlenmeyer flask were added 200 cc. 0·25 *N* sodium hydroxide[1] at 37°. The flask was set in a thermostat at 37° and suction apparatus so arranged that air could be slowly bubbled through concentrated sulphuric acid, then through the lecitho-vitellin solution and finally through 200 cc. *N*/100 HCl. The protein was not completely dissolved until the sixth hour of the experiment. Every 12 hours, from the time the protein solution was placed in the water-bath, the following aliquots were taken for analysis.

[1] Prepared from sodium and found to contain no iron.

1. 15 cc. of the protein digest were slowly added to 50 cc. of 3·5 % trichloroacetic acid[1] in a centrifuge-tube, and the heavy white precipitate was separated.

(*a*) A 40 cc. aliquot of the clear supernatant liquid was taken for the iron estimation.

(*b*) A micro-Kjeldahl nitrogen determination was made on a 2 cc. aliquot of the supernatant liquid.

(*c*) The trichloroacetic acid precipitate was dissolved in the minimum amount of dilute sodium hydroxide and reprecipitated with trichloroacetic acid. The entire precipitate was ashed and the iron content determined.

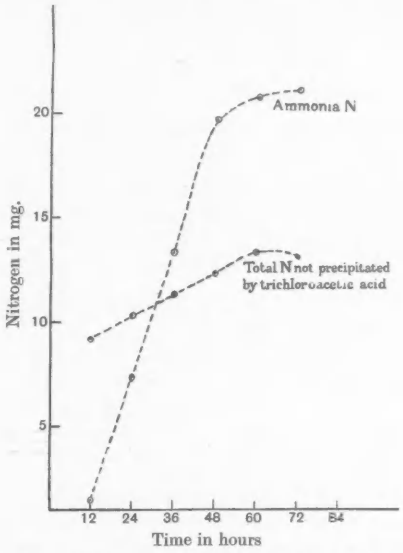

Fig. 1. Action of 0·25 *N* NaOH on lecitho-vitellin.

2. Amino-nitrogen (micro-Van Slyke) was determined on a 2 cc. aliquot of the protein digest.

3. 10 cc. of the hydrochloric acid solution were titrated with *N*/100 NaOH, using alizarin-red indicator. The ammonia formation during hydrolysis was thus determined.

During the action of 0·25 *N* NaOH on lecitho-vitellin ammonia is slowly evolved and the amount of nitrogen not precipitated with trichloroacetic acid steadily increases (Fig. 1). The two values reach maxima at the same time, *i.e.* after about 60 hours' digestion. The first determination of free amino-nitrogen showed 0·98 mg. in 2·0 cc. of the hydrolysate. This value was not increased up to 48 hours, at which time the determination was discontinued. The results of the micro-iron determinations are shown in Table I.

[1] The trichloroacetic acid was found to contain iron which was removed by distilling the acid.

Table I.

Time hydrolysed with 0·25 *N* NaOH hours	Amount of iron recovered: Supernatant liquid mg.	Amount of iron recovered: Trichloroacetic acid precipitate mg.	Total amount of iron in 15 cc. of the hydrolysate: Found mg.	Total amount of iron in 15 cc. of the hydrolysate: Calculated* mg.
6	0·042	0·032	0·099	0·072
12	0·060	0·007	0·104	0·072
24	0·209	0·002	0·302	0·072
48	0·396	0·002	0·515	0·072

* The lecitho-vitellin contained 0·047 % iron.

Note. The iron content of the supernatant liquid after 12 hours' digestion was greatly increased, from some source other than the lecitho-vitellin. The solution at this time gave an intense red colour with potassium thiocyanate. The source of this inorganic iron was undoubtedly the glass flask.

The values for the iron precipitated by trichloroacetic acid show clearly that after 12 hours' digestion the whole of the lecitho-vitellin iron has been liberated from protein combination. Only a trace of iron (less than 3 % of the original iron content of the protein) was contained in this precipitate after 24 hours' digestion. Repetition of this experiment, using quartz ware, was therefore not considered to be necessary at present. At the completion of the digestion (72 hours) a more detailed study of the products formed from lecitho-vitellin by mild hydrolysis with 0·25 *N* NaOH was undertaken. A preliminary experiment showed that after the precipitation of the protein with trichloroacetic acid the filtrate contained a compound or compounds which could be precipitated from acid solution by a saturated solution of normal lead acetate. This lead salt was decomposed with hydrogen sulphide, and the excess hydrogen sulphide in the filtrate from the lead sulphide was driven off by boiling. This filtrate gave an intense biuret reaction.

In the following experiment the distribution of nitrogen and iron in these fractions of lecitho-vitellin, formed by hydrolysis with dilute alkali, was determined. Aliquots of the filtered hydrolysate were separated into the following fractions and the nitrogen content of each determined by micro-Kjeldahl.

I. *The partition of nitrogen.*

A. 30 cc. of the protein digest (total nitrogen = 55·2 mg.) were added to 50 cc. 3·5 % trichloroacetic acid.

Filtrate (1)
Nitrogen content = 27·2 mg., *i.e.* 47·6 % of the total nitrogen. To 50 cc. of this solution (10·12 mg. nitrogen) were added 10 cc. saturated normal lead acetate.

Precipitate (1)
Nitrogen content = 28·28 mg., *i.e.* 51·20 % of the total nitrogen.

Filtrate (2)
Nitrogen content = 6·52 mg., *i.e.* 11·8 % of the total nitrogen.

Precipitate (2)
Decomposed by H_2S, *etc.* (see above). Nitrogen content = 4·80 mg., *i.e.* 8·7 % of the total nitrogen.

B. 25 cc. of the protein digest (total nitrogen = 46·0 mg.) were neutralised with 0·25 *N* acetic acid.

Filtrate (100 cc.)	Precipitate
Nitrogen content = 28·4 mg., *i.e.* 61·7 % of total nitrogen.	Nitrogen content = 16·7 mg., *i.e.* 36·3 % of total nitrogen.

On adding saturated normal lead acetate to this filtrate a heavy white precipitate was formed. We were unable to separate this precipitate quantitatively, the filtrate being always slightly cloudy. 50 cc. of this filtrate were, however, precipitated with saturated normal lead acetate and the precipitate decomposed with H_2S, *etc.* (see above).

II. *The iron distribution.*

The trichloroacetic acid precipitate (1) contained little or no iron. The lead acetate precipitate (2) contained the iron in organic combination. The filtrate (2) contained a large amount of ferric iron. The source of this inorganic iron has already been discussed. The substance precipitated on neutralising the hydrolysate with acetic acid contained only a trace of iron. The quantitative results were as follows:

	Iron content mg.
A. Lead acetate precipitate (2) from 50 cc. filtrate (1)	0·082
∴ 80 cc. (approx.) trichloroacetic acid filtrate (1) should contain	0·13
∴ 30 cc. of the original protein hydrolysate contain	0·13
30 cc. of the original protein hydrolysate contain (calculated)	0·14

	Iron content mg.
B. Lead acetate precipitate from 50 cc. acetic acid filtrate	0·071
∴ The acetic acid filtrate (100 cc.) should contain approximately	0·14
∴ 25 cc. of the original protein hydrolysate contain	0·14
25 cc. of the original protein hydrolysate contain (calculated)	0·12

Note. 200 cc. of the original lecitho-vitellin solution contain 0·94 mg. Fe (lecitho-vitellin contains 0·047 % Fe). Allowing for the change in volume from evaporation (measured) during the 72 hours' duration of the digestion 30 cc. of the hydrolysate should contain 0·14 mg. Fe.

It may be concluded from these results that the substance or substances precipitated by normal lead acetate from the trichloroacetic acid filtrate, or from the acetic acid filtrate contain the lecitho-vitellin iron in organic combination.

Copper in lecitho-vitellin.

In the course of investigations on nutritional anaemia we planned to use lecitho-vitellin or its iron-containing fraction as dietary sources of "organic" iron, provided these substances contained no copper. This sample of lecitho-vitellin when analysed by the procedure recommended by McFarlane [1932, 1] was found to contain copper. The amount of copper present, compared with the amount in biological materials in general, was surprisingly high. At this time Dr R. C. Hamilton in this laboratory had prepared another sample of

lecitho-vitellin by essentially the same procedure as already described. The two samples were analysed with the following results:

Sample of lecitho-vitellin	Copper %	Iron* %	Ratio Cu : Fe
McFarlane	0·0034	0·047	1 : 13·8
Hamilton	0·0032	0·045	1 : 13·4

* After decomposition of the organic substance the iron was determined by potentiometric titration with titanous sulphate [see McFarlane, 1932, **1**].

Egg-yolk, according to McFarlane, Fulmer and Jukes [1930], contains approximately 2·5 g. of protein and about 0·08 mg. copper (average value from their Table IX). They found, however, that by changing the protein in the diet the total protein content of the yolk could be varied as much as 30 %. The copper content of the entire egg was also found to be an extremely variable quantity. Livetin constitutes about one-fifth of the total yolk-protein, according to Kay and Marshall [1928], and the total amount appears to be relatively constant from one egg to another. Assuming that these average values represent approximately the total protein, livetin and copper contents of the egg-yolk; that the lecitho-vitellin contains all the copper, and that the yolk contains only these two proteins, we find:

Average copper content of yolk	0·08 mg.
„ total protein (vitellin and livetin)	2·5 g.
„ amount of livetin	0·5 g.
Vitellin content of yolk	2·0 g.

The copper content of lecitho-vitellin should be approximately

$$\frac{0{\cdot}08}{2{\cdot}0} \times 100 = 4{\cdot}0 \text{ mg./100 g.}$$

Loeschke [1931] has obtained more constant values for the copper content of the hen's egg (0·055–0·095 mg.) than those recorded by McFarlane, Fulmer and Jukes. The copper content of lecitho-vitellin, using the average of Loeschke's values, should be about

$$\frac{0{\cdot}066}{2{\cdot}0} \times 100 = 3{\cdot}3 \text{ mg./100 g.}$$

These calculated values are probably only rough approximations. Since they are, however, of the same order of magnitude as the actual copper content of lecitho-vitellin, it is not improbable that all the copper of the yolk is contained in this protein.

Some preliminary observations on the behaviour of the copper when lecitho-vitellin is hydrolysed by 0·25 *N* NaOH at 37° have also been made. In the following separations precautions were taken to avoid copper contamination from reagents, glassware, *etc.* All filtrations were made with Jena glass filters.

5 g. lecitho-vitellin were boiled with absolute alcohol for 24 hours. The ash of the alcohol-soluble material contained copper. The amount, too small to determine, must have been less than 0·02 mg. and its presence in this fraction was therefore not considered significant. The vitellin, which was now

pink in colour[1], was hydrolysed with 500 cc. 0·25 *N* sodium hydroxide at 37° for 72 hours. A very small amount of insoluble material was filtered from the digest. The theoretical amount of glacial acetic acid required to neutralise the hydrolysate was added from a burette, drop by drop with constant stirring. A heavy white precipitate formed, which was allowed to settle before filtering. (The acetic acid precipitation is incomplete.) The filtrate was evaporated to a volume of about 100 cc. and the precipitation of the protein completed with trichloroacetic acid.

The two protein fractions were combined, dissolved in the minimum amount of dilute sodium hydroxide and reprecipitated with trichloroacetic acid. The solution and precipitation were repeated once more and the protein fraction finally dried with absolute alcohol. This fraction was also found to contain copper. The amount of copper in 1 g. of the protein was again too small to be accurately determined.

To the protein-free filtrate was added a saturated solution of normal lead acetate and a copious white precipitate formed. The precipitate was decomposed with hydrogen sulphide and the filtrate from the lead sulphide precipitate boiled to remove the hydrogen sulphide. The solution was transferred to a 50 cc. volumetric flask and diluted to volume. This solution was colourless and gave strong biuret, Millon's and glyoxylic acid reactions. It contained a considerable amount of phosphorus, a trace of mercaptan sulphur, and gave a white precipitate with alcohol. This solution was analysed with the following results:

Wt. of substance (or substances) isolated, 0·5440 g.

	% of dry substance
Nitrogen (micro-Kjeldahl)	9·06
Total ash	4·24
Iron [McFarlane, 1932, 2]	1·23
Copper [McFarlane, 1932, 1]	0·0082

Assuming complete precipitation by normal lead acetate, this fraction represents approximately 10 % of the lecitho-vitellin. Only about 25 % of the total copper in lecitho-vitellin was recovered in this fraction. The copper content of the substance or substances isolated is tentatively fixed at 0·008 %. The filtrate from the lead acetate precipitate gave a strong biuret reaction and contained about 10 % of the original lecitho-vitellin nitrogen. Whether the presence of copper in this filtrate is due to incomplete precipitation with lead acetate or to the presence of a second copper-containing fraction in lecitho-vitellin has still to be determined. The presence of lead in this filtrate interferes with the reagent we have used (sodium diethyldithiocarbamate) to detect or determine copper.

The above observations are obviously of a preliminary nature. The composition of this lead acetate precipitate is being investigated.

[1] It was of interest to note that the 72-hour hydrolysate of vitellin (alcohol-extracted lecitho-vitellin) had a fairly deep reddish-brown colour while the same digest of lecitho-vitellin had only a faint yellowish tinge.

Discussion.

During the action of 0·25 *N* sodium hydroxide on lecitho-vitellin an organic iron-containing substance is rapidly liberated from protein combination. The decomposition is accompanied by a more gradual evolution of ammonia and a steady increase in the amount of non-protein nitrogen formed. From the difference in the rates of these reactions it appears unlikely that the latter process is related in any way to that involving the liberation of the iron-containing fraction. No corresponding increase in free NH_2 groups was found. The amount of amino-nitrogen (Van Slyke) was not increased during the hydrolysis.

At the completion of hydrolysis a protein substance can be precipitated by trichloroacetic acid or by neutralising the hydrolysate with acetic acid. This precipitate contains a trace of iron and of phosphorus, the amount of which decreases with repeated precipitation. There is a striking similarity in the action of dilute sodium hydroxide on lecitho-vitellin and on caseinogen.

The filtrate from the trichloroacetic acid precipitate contains about 50 % of the lecitho-vitellin nitrogen. Part of this non-protein nitrogen (about 20 %) is contained in a compound, or compounds, which can be precipitated from acid solution by normal lead acetate. This precipitate contains the lecitho-vitellin iron and part of the copper (approximately 25 %) so combined that no colour is given directly with the sodium diethyldithiocarbamate reagent of Callan and Henderson [1929].

Whilst we have not yet investigated the composition of this lead salt (or salts) in any detail, we have found that some of its properties, particularly so far as its solubility is concerned, differ from those of haematogen. In this respect it resembles more closely the vitellinic acid of Levene and Alsberg [1900] but contains more iron (1·23 %) than that recorded for vitellinic acid (0·57 %).

Having found that the total copper content and the ratio of copper to iron were practically the same in two samples of lecitho-vitellin prepared by different workers, we felt justified in concluding that copper was a constituent of the vitellin molecule. It still remained to be proved, however, that the copper is in chemical union with the vitellin. These results might possibly be explained from the standpoint of adsorption since organic substances and particularly proteins have a great affinity for copper.

We have found that:

1. At least part of the copper of vitellin (25 %) is contained in a fraction which has been separated by precipitation with normal lead acetate.

2. The copper is contained in the filtrate after decomposing the precipitate with hydrogen sulphide in acid solution.

3. The copper is so combined that it does not react with sodium diethyldithiocarbamate, a reagent which will detect less than 0·1 part per million of copper [McFarlane, 1932, 1].

It now appears justifiable to conclude that the copper is present in chemical combination with an organic substance. This copper is present in a substance,

rich in iron, which is generally conceded to be the mother substance of the haemoglobin formed by the avian embryo. It is, therefore, suggested that in the lecitho-vitellin molecule there is a component containing iron and copper in organic combination, which possibly represents, at least in the chick, an intermediate stage in the formation of haemoglobin. In the present state of our knowledge, any further discussion of the possible significance which this fact may have in regard to the action of copper in stimulating haemoglobin synthesis can more appropriately be reserved for a future communication.

Hill [1931] has recently submitted evidence which indicates that the iron of egg-yolk is present as free ferric iron rather than in any stable organic combination, since egg-yolk suspended in acetate buffer does not give the reaction of ferrous iron with $\alpha\alpha'$-dipyridyl but on adding sodium hydrosulphite a deep red colour is immediately developed. Quantitative determinations of the total iron in egg-yolk by the direct pyridyl reaction, using sodium hydrosulphite and sulphur dioxide or 30 % alcohol, gave results almost identical with those obtained after incineration of the egg-yolk. Needham [1931] accepts these experiments of Hill as disproof of the earlier conclusions of Bunge and Hugounenq and Morel that the iron of haematogen was not merely adsorbed inorganic iron.

All the iron of egg-yolk is not indeed contained in haematogen, and it is not improbable that colloidal ferric hydroxide may also be present. McFarlane *et al.* [1930] have found the iron contents of 38 samples of the total egg-yolk-protein to vary from 0·031 to 0·066 % (average 0·045 % Fe). Taking the average weight of egg-yolk-protein recorded by Kay and Marshall, egg-yolk should contain:

Total protein	4·5 g.
% iron in protein	0·045
Total iron in the yolk-protein	0·002 g.
Total iron content of yolk [Sherman, 1927]	0·0086 g.
„ „ [Hill, 1931]	0·0085 g.

Fresh egg-yolk, according to Aggazzotti [1913], has a p_H of about 4·5, while Healy and Peter [1925] record p_H 6·2 to 6·6. The presence of iron as ferric hydroxide is quite possible at the latter p_H but improbable at p_H 4·5. The qualitative reaction with $\alpha\alpha'$-dipyridyl and egg-yolk in the presence of a reducing agent indicates that at least part of the non-vitellin-iron is present as colloidal ferric hydroxide.

Needham [1931] and Elvehjem [1932] have concluded from Hill's results that egg-yolk contains only inorganic iron. This interpretation of Hill's results involves several very debatable assumptions which the author himself did not choose to make. In his summary Hill says "egg-yolk contains no detectable organic iron analogous to a haematin compound." This, we believe, is a logical interpretation of his quantitative results and of the fact that haematin in the presence of the reducing agent does not react with $\alpha\alpha'$-dipyridyl.

To consider that these findings constitute proof that the iron in egg-yolk is all in inorganic form is to assume that if egg-yolk contains iron in organic

combination the iron linkage must be the same as that in the haematin molecule. What little evidence we have suggests that in vitellin the iron is less firmly combined than the iron in the haematin molecule. Lintzel [1928] has shown that haemoglobin is extremely resistant to digestion and is not utilised by the organism. According to Elvehjem [1932], the iron of haematin, in contrast with ferric chloride, gave a poor response in haemoglobin production when fed to anaemic rats.

We could find no evidence that $\alpha\alpha'$-dipyridyl reacts only with inorganic ferrous compounds and does not combine with ferrous organic compounds other than reduced haematin. The ferrous salt of $\alpha\alpha'$-dipyridyl, according to Blau [1898], is $[Fe(C_{10}H_8N_2)_3]X_2$. X may represent a univalent inorganic or organic anion. It is thus possible to have compounds of the type $[Fe(C_{10}H_8N_2)_3]R_2$, where R is a negative organic radical.

From our own observations on the cleavage of the vitellin molecule by relatively mild chemical treatment it is reasonable to presume that a strong reducing agent such as sodium hydrosulphite might bring about the same decomposition much more rapidly, *e.g.*,

(1) Vitellin $\xrightarrow{0{\cdot}25\ N\ NaOH}$ Protein + FeR_3 (R = organic anion);

(2) Vitellin $\xrightarrow{Na_2S_2O_4}$ Protein + FeR_2, and with dipyridyl an addition compound may be formed;

(3) $FeR_2 + 3(C_{10}H_8N_2) \rightarrow [Fe(C_{10}H_8N_2)_3]R_2$.

The compound finally formed might have a red colour and be soluble in alcohol or extracted from a protein adsorption compound by sulphur dioxide. Since the red colour is probably due to the complex ferrous cation, it is not likely that substitution of an organic anion for an inorganic anion would affect the colour of the compound.

Summary.

1. Some preliminary experiments have shown that during the action of 0·25 *N* NaOH on lecitho-vitellin at 37° there is a gradual evolution of ammonia and a steady increase in the amount of non-protein-nitrogen but no increase in free amino-groups. At the completion of hydrolysis (60–70 hours) the hydrolysate had the following properties.

(*a*) Trichloroacetic acid, or neutralisation with acetic acid, precipitates a protein substance which contains about 50 % of the lecitho-vitellin-nitrogen but does not contain iron or phosphorus.

(*b*) Part of the non-protein-nitrogen (about 20 %) is contained in a compound (or compounds) which is precipitated from acid solution by normal lead acetate.

2. Two samples of lecitho-vitellin prepared by different workers contained practically the same amount of copper (0·0033 %) and iron (0·046 %). The iron

and copper are believed to be contained in the vitellin molecule in firm organic union for the following reasons.

(*a*) All the iron and at least 25 % of the copper are contained in the normal lead acetate precipitate.

(*b*) The iron and copper are contained in the filtrate after decomposition of the lead salt, or salts, by hydrogen sulphide. This solution gives no direct reaction with potassium thiocyanate or sodium diethyldithiocarbamate. The latter reagent will detect less than 0·1 part per million of copper.

(*c*) The iron and copper contents of this fraction are tentatively fixed at 1·23 and 0·0082 % respectively of the dry weight of substance.

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CXXV. THE TRANSMISSION OF VITAMIN A FROM PARENTS TO YOUNG IN MAMMALS.

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THE attack now being made on the important question of the vitamin A reserves of the human organism in relation to health and disease [Wolff, 1929] may be expected to advance considerably our knowledge of the possible benefits of vitamin A therapy; but whatever may be learned of the influence of the vitamin A reserve of the adult upon health, it is unlikely that work with uncontrolled material in the form of available tissues from autopsies can yield much information concerning the importance and effect of the vitamin A supply of the foetus and the growing child before and after weaning. Wolff has noted that, weight for weight, the livers of new-born children examined by him contained much less vitamin A than the livers of adults: 62·5 % contained no vitamin A, 25 % contained up to one-half as much as the average adult, and 12·5 % contained as much as the average adult examined by him. Although this observation was based on the examination of a small number of livers, it does suggest that the infant is normally born with only a small store of vitamin A in its liver and it directs interest to the factors which control the supply of vitamin A to the child up to the time of weaning, knowledge of which will show how the vitamin A store of the infant can best be enriched. As the controlled experimental work which is needed for an investigation of these factors cannot be performed on human beings, the interest is at present transferred to lower mammals, for which the relevant conditions are essentially similar.

There are two ways in which a young mammal may receive an endowment of vitamin A before it is weaned. The vitamin A may pass through the placental barrier into the foetal circulation during gestation, or it may be ingested by the suckling in the colostrum and the milk. Available data concerning this transmission of vitamin A from mother to young are scanty. It has not been recorded that the vitamin can pass the placental barrier of any other mammal than the human, and the factors affecting the passage in the human are nowhere mentioned, so far as the writer is aware. It is known that vitamin A is present in human, cow's and goat's milk, but again the factors which control the amount in the milk are unknown. Drummond, Coward and Watson [1921] claimed that the vitamin A content of cow's milk is closely dependent on the vitamin content of the diet and shows seasonal variation; but

recently MacLeod, Brodie and MacLoon [1932] have found that there is little seasonal variation in the vitamin A content of cow's milk, and inferred that the vitamin content of the diet has little effect on that of the milk. Moore [1932] has calculated that the amount of carotene ingested by the cow in summer pasture is many times greater than that secreted with the milk-fat as vitamin A or unchanged carotene, while much vitamin A is stored in the liver and an even greater amount of carotene excreted unchanged. Drummond, Coward and Watson [1921] also reported that cow's colostrum is richer in vitamin A than cow's milk.

Experiments on the effect of the mother's diet on the vitamin A reserves of the young rat at weaning were performed by Scheunert and Candelin [1925] who claimed that an increase in the mother's intake of vitamin A caused an increase in the vitamin A reserves of the young; but apparently the young rats had access to solid food containing vitamin A after they were able to eat, so that it is doubtful whether the authors were justified in drawing any conclusions from the results of the experiments they described.

The fullest use of the data mentioned does not, however, enable us to gain an insight into the factors controlling the transmission of vitamin A from the mammalian mother to her young, although it does suggest that any such transmission is a strictly regulated process. In the principal experiment described below a quantitative study has been made of the effect of the carotene content of the maternal diet before and during lactation upon the amount of vitamin A received by young rats up to the time of weaning, in order to obtain further information about the vitamin transfer.

Experimental.

The presence of vitamin A in the liver of embryo rat and rabbit.

Before proceeding to quantitative experiments on the transmission of vitamin A it was thought advisable to find whether the liver of the embryo normally contains vitamin A in the species to be used. Two foetuses of the rat (taken at random from females in the stock colony) were killed at birth and the liver was dissected from each, weighed, and dissolved in 5 cc. of 5 % aqueous KOH. The solution was extracted with ether, and the ethereal solution dried by filtering through anhydrous sodium sulphate and evaporated. The oily residue was dissolved in 1 cc. of chloroform and 0·5 cc. of the solution treated with 2 cc. of antimony trichloride reagent. A faint blue colour showing absorption at 620 $m\mu$ was produced, indicating the presence of some vitamin A in the tissue. The remaining 0·5 cc. of solution of the liver oil was then treated with antimony trichloride and the depth of colour measured in a Lovibond tintometer. It was thus found that the whole liver of each foetus contained 10 B.U. (blue units) of vitamin A, corresponding closely to 30 B.U. per g. of liver tissue. (All figures recorded for the colorimetric estimation of vitamin A are expressed in Lovibond blue units calculated by the method of Moore [1930].)

Examination was also made of the liver oils of still-born foetuses, which gave about the same figure for total vitamin A content; and of the whole body fat of the foetus, which had the same total vitamin A content as the liver oils alone. The fat from the foetus from which the liver had been removed was found to contain no vitamin A, showing that the small vitamin A store of the foetus is chiefly contained in the liver—a distribution similar to that in the adult. The presence of vitamin A in the body of the embryo before term was also demonstrated by killing a pregnant rat at 15 days and removing the embryos from the uterus. Two were dissolved whole in 5 cc. of 5 % aqueous KOH and treated as above, when it was found that each embryo contained 5 B.U. of vitamin A.

Similar observations were made with the livers of newborn and embryo rabbits. As an average figure for several newborn rabbits, chosen at random from different stock litters, each whole liver contained 100 B.U. of vitamin A, corresponding to 25 B.U. per g. Two embryos were taken from a doe four weeks pregnant and the liver of each was found to contain 30 B.U. of vitamin A. Thus the embryo receives from the parental organism through the placental barrier either vitamin A or materials from which to make its own vitamin A in the rat and the rabbit as well as the human.

Transmission of vitamin A to the embryonic and suckling rat.

The chief experiment was carried out on a single group of females, the controlled variables of the experiment being (*a*) the vitamin A content of the diet from weaning to the end of pregnancy and (*b*) the vitamin A content of the diet during lactation. Measurements were made of (*a*) the vitamin A content of the livers of some of the newborn young from each doe's litter at birth, (*b*) the vitamin A content of the livers of some young from each doe's litter at weaning, (*c*) the vitamin A content of the doe's liver immediately after she weaned her litter, and (*d*) the growth of the remaining young rats from each litter when fed on a vitamin A-free diet after weaning.

The group of females for experiment was chosen from a single litter of Glaxo B stock albinos containing 9 does and 2 bucks. The does were labelled *A* to *I* and separated into three groups at four weeks old, when all nine weighed between 34 g. and 39 g. Does *A* and *B* were placed on a vitamin A-free diet and given no supplement to it until they had ceased to grow; thereafter they received a small daily dose of vitamin A, sufficient to maintain a submaximal growth rate. Does *C*, *D* and *E* were given a diet composed of maize meal 1060, milk powder 400, whole grain wheat 200, dried yeast 200, wheat germ 60, calcium phosphate 60 and salt mixture 20 parts, with a bi-weekly supplement of a little lettuce and milk. Does *F*, *G*, *H* and *I* received a basal diet containing 90 % of the mixture fed to *C*, *D* and *E* and 10 % of red palm oil, with a similar supplement of lettuce and milk. Thus the animals *A* and *B* received a diet very low in vitamin A, while *C*, *D* and *E* received a moderate

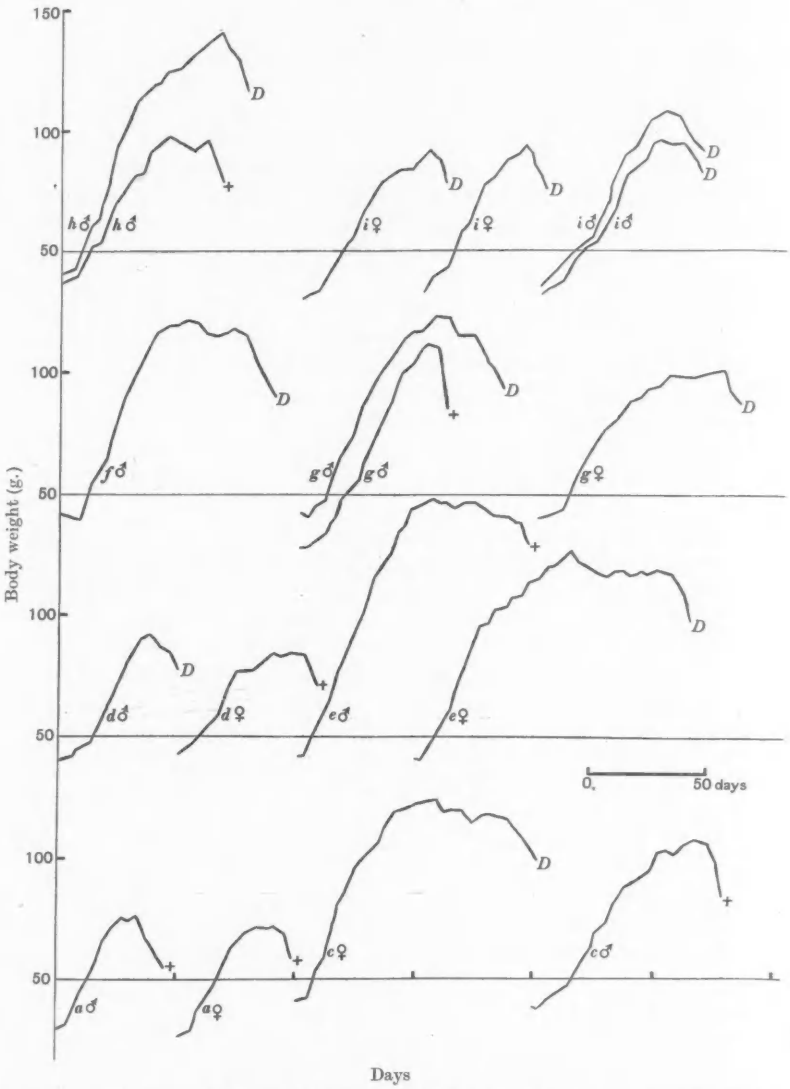

Fig. 1. Growth curves of young rats from does fed with different amounts of carotene during pregnancy and lactation. (For diet of parents compare Table I.) + signifies died. *D* signifies discontinued. *a, c, d, etc.* signify offspring of doe *A, C, D, etc.*, respectively.

amount, and *F*, *G*, *H* and *I* a large amount of carotene. When the does were three months old a buck was introduced to the cage housing each group, and each doe when seen to be pregnant was removed to a separate cage but kept on the same diet until the birth of her litter. All the does except *A* and *B* grew fairly uniformly and weighed between 220 g. and 250 g. when they produced litters; doe *B* died and doe *A* grew more slowly than the others and bore a litter when weighing 180 g. After the birth of the litters the diets were rearranged as follows: the does *A*, *C*, *D*, *F*, *G* received the vitamin A-free diet and the does *E*, *H*, *I* received the red palm oil diet rich in carotene. To ensure that none of the young should ingest vitamin A except in the milk, all does receiving the red palm oil diet were fed in a cage apart from the litter and brushed before being returned to the litter cage.

At the birth of each litter two young were killed (only one if the litter was small) and the liver of each dissected out; the liver oils were examined for vitamin A as described above. Each litter was weaned at the age of 21 days and two young were killed in order to examine the liver oils for vitamin A; the remaining animals were given a diet of white bread moistened with water for one week and afterwards a synthetic vitamin A-free diet. Their weight curves were plotted until they were depleted of the vitamin. After the weaning of the litter the doe was generally killed also in order to measure the store of vitamin A in her liver. These measurements are collected in Table I and the growth curves of the young rats weaned from the experimental does are shown in Fig. 1.

Table I. *Vitamin A transfer from mother to young in the rat.*

Amounts of vitamin A are expressed in Lovibond blue units (see text).

Parent doe	*A*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Carotene content of parent's diet from weaning to birth of litter	V. low	Medium	Medium	Medium	V. high	V. high	V. high	V. high
Carotene content of parent's diet while suckling litter	None	None	None	V. high	None	None	V. high	V. high
Amount of vitamin A in liver of young at birth	0	5	10	5	10	10	0	10
Amount of vitamin A in liver of the young at weaning	0	30	10	75	35	30	66	25
Amount of vitamin A in liver of parent after weaning young	20	—	1125	—	—	22,000	20,000	10,000
Average weight of young at weaning (g.)	28	40	40	42	42	35	40	32
Maximum weight attained by young on A-free diet after weaning (g.)	74	118	89	140	122	112	122	98
Days on A-free diet taken to deplete vitamin A store of young	50	77	50	78	79	62	66	55
Number of young weaned	4	4	5	4	2	5	4	6

Transmission of vitamin A to the embryonic rabbit.

An observation was made upon the effect of the carotene content of the diet of the rabbit during pregnancy on the amount of vitamin A stored in the liver of the young at birth. From a breeding colony two does were selected of similar age, weight and dietetic history. After mating, one of them received

the ordinary diet of the colony; the other was fed on bran, which had been rubbed with red palm oil, and occasional meals of cabbage to sustain its appetite. During pregnancy it consumed about 3 kg. of bran and 300 g. of red palm oil.

At the birth of the litters two young were removed from each and their livers dissected out. The liver oils were examined in the usual way for vitamin A and it was found that the vitamin A content of each whole liver was about 100 B.U. There was no additional amount of vitamin A in the livers of the young whose parent had received the additional carotene. From this finding it is concluded that the rabbit resembles the rat in that the amount of vitamin A in the liver of the young at birth is not dependent on the amount fed to the mother, provided that she has some reserve.

Discussion.

Deductions from the quantitative data on vitamin A transmission in the rat.

From the figures given in Table I the following conclusions may be drawn concerning vitamin A transmission to the young rat.

1. The amount of vitamin A in the liver of the rat at birth is small and subject to little variation. It cannot be increased by giving large amounts of carotene to the mother before or during pregnancy.

2. The amount of vitamin A in the liver of the young rat when it is weaned is generally larger than the amount in its liver at birth, and is more variable. It is dependent on the carotene content of the mother's diet during lactation, which is evidently a factor in determining the amount of vitamin A in the milk. Thus, under the conditions described, the vitamin A store in the liver of the young rat increased on the average during suckling by 50 B.U. when the lactating mother received a diet rich in carotene, but only by 12 B.U. when the mother received a diet devoid of carotene and vitamin A. When the mother had a negligible reserve of vitamin A in her liver and received a diet free from carotene during pregnancy and lactation, no measurable amount of vitamin A could be found in the liver of the young at weaning (see figures for doe *A* in Table I). It is probable that the vitamin A reserve of the mother at parturition also exerts an effect on the vitamin A content of the milk but it must be considerably smaller than the effect due to carotene in the diet during lactation, and is not large enough to show up unequivocally in the work described.

3. The weight of the young rat at weaning does not appear to be greatly influenced by the amount of carotene received by the mother at any time, with the exception that the offspring of a doe almost depleted of vitamin A may be stunted (see doe *A*, Table I).

4. The maximum weight attained by the young and the period for which they can subsist when placed on a vitamin A-free diet after weaning do not appear to depend on the amount of carotene received by the mother before or during lactation; again with the exception that if the mother is almost depleted

and receives a vitamin A-free diet during lactation the growth and the period will both be restricted.

It must be emphasised that for the present purpose whenever vitamin A was found in the foetal liver the assumption was made that this vitamin was obtained from the mother by transmission through the placental barrier. Two other possibilities demand attention. First, it is possible that carotene and not vitamin A passes from the mother to the foetus; a possibility which cannot at present be tested with the rat as the carotene would presumably be transformed into vitamin A in the foetal liver. It may be remarked that the liver oils of the foetus are almost completely colourless and can therefore contain no carotene. Secondly, there is a possibility that the foetus may at some stage of its development be able to synthesise vitamin A from simpler substances. This, however, would appear to be improbable in view of the observation that the foetus is generally born with little vitamin A in its liver and sometimes with none at all.

As the vitamin A store of the young rat cannot be raised by feeding large amounts of carotene to the mother during pregnancy and lactation, there must be some factor at work limiting quite strictly the amount of vitamin A which can pass through the placental barrier or into the milk. Gunderson and Steenbock [1932] have found that "increasing the vitamin B intake both absolutely and in relation to the amount of milk secreted had no discernible effect on the vitamin B content of cow's or goat's milk" and concluded that "the maximum vitamin B content of milk is under definite physiological control." Their finding appears to be an exact parallel with that now reported for vitamin A, but one hesitates to postulate a "physiological control" of transmission to the foetus and into the milk. In the first place, it has been emphasised by Moore [1932] that there is a similar "control" of the maximum vitamin A content of cow's milk, and that while the amounts of carotene and vitamin A passing into the milk daily are very small compared with the intake of carotene, large amounts of vitamin A are stored by the liver. A simple explanation of this would be that the carotene after entering the blood-stream is nearly all absorbed in its passage through the liver, so that little would be found in the general circulation.

A point which emerges from the data of Table I and requires further investigation is that there is no simple proportionality between the vitamin A store of the young at weaning and the length of time they can subsist on a vitamin A-free diet after weaning: thus a rat having a vitamin A store of about 70 B.U. was able to live for 70 to 80 days, one with a store of 25–30 B.U. for about the same time, while one with no measurable store of vitamin A lived for 50 days before declining in weight. It is known that the vitamin A store in the liver of the young rat has a considerable effect on the length of time required to deplete its store on a vitamin A-free diet, but the form of the relationship is by no means clear. It is receiving attention in further work.

In the work described no attempt was made to distinguish between the

colostrum and milk of the rat, so that all references to transmission of vitamin A in the milk include the colostrum; it must be borne in mind that the colostrum may differ from the milk in vitamin A content.

Application of findings to other species.

It is not to be expected that the same findings would necessarily be obtained for other species of mammals. The complexity of the placenta varies from species to species so that in the more highly developed forms, as for example in man and the rat (haemochorial placenta), the maternal and foetal bloods come into much more intimate contact than in less highly developed forms such as those of the cow or goat (syndesmochorial placenta) or the pig (epitheliochorial placenta). When the contact between the two circulations is more intimate more substances are transmissible through the placental barrier than when the contact is less intimate. Whether carotene or vitamin A would pass with equal facility through all types of placenta is unknown and must remain for the present an open question.

Needham [1931, p. 1497] has pointed out a relationship between the placenta and the colostrum—namely that in species with more highly developed placentas the colostrum has less importance and is more like the later milk than in species with less highly developed placentas. From this it would appear to be likely that, although the colostrum of the cow is richer in vitamin A than its milk, the same may not hold true for man or the rat. A further investigation of this point is in progress.

Since man and the rat both possess placentas of the same type it may be assumed that the relative importance of the placenta, colostrum and milk for the transmission of vitamin A from mother to young will be similar for the two species. On the basis of this assumption the quantitative results described above suggest that the best way of applying vitamin A therapy with the object of giving the child a reserve of vitamin A is to dose it directly. Failing this, the best results may be expected to follow from giving the mother regular fairly high doses of vitamin A during the period of suckling. To give the mother vitamin A during pregnancy will not affect the vitamin store of her child to any noticeable extent.

Summary.

1. It has been shown that vitamin A is normally found in the liver of the rat and the rabbit at birth, but only in small amount which cannot be increased by giving the mother a diet rich in carotene during gestation.

2. The store of vitamin A in the liver of the young rat increases two- or three-fold during suckling, but the increase may be greater when the lactating parent receives a diet rich in carotene. There appears to be a limit to the amount of this increase, due in turn to a limitation of the amount of vitamin A which can pass into the milk.

3. The significance of these results for other species is discussed and the conclusion reached that in human beings direct feeding of the vitamin to the infant must be employed to build up its reserve of vitamin A.

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CXXVI. STUDIES ON THE CHEMICAL NATURE OF SECRETIN.

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Although it is now thirty years since secretin was first discovered by Bayliss and Starling [1902] very little is known as yet of its chemical nature. This is largely due to the fact that it is only comparatively recently [Weaver, Luckhardt and Koch, 1926; Mellanby, 1928] that the hormone has been separated completely from the depressor substance that is always present in acid extracts of the duodenum, as prepared by the original method of Bayliss and Starling, and obtained in a state approaching purity. The recent products of Mellanby [1928], Still [1930], and Ivy *et al.* [1930] have been shown by animal experiments to be physiologically pure, but chemical purity has not been claimed in any case.

Even before secretin had been separated from the depressor substance—vaso-dilatin—some evidence that it might possess a peptide character was obtained by Bayliss and Starling, who found that it was destroyed by proteolytic enzymes, and by hot acids and alkalis. They also showed that the molecule was dialysable. Lalou [1912] confirmed this observation and also showed that it passed through a thick collodion membrane.

Mellanby and Huggett [1926] suggested that secretin was a secondary proteose, since they found that it was precipitated from solution by complete saturation with ammonium sulphate, but not by half saturation. Later, Mellanby [1928] modified these views, suggesting that it was a polypeptide with a high organic phosphorus content. This was disputed by Takács [1928, 1, 2], Still [1930] and Ivy *et al.* [1930], all of whom claimed to have prepared highly active concentrates that were phosphorus-free. The presence of sulphur was reported by all these workers.

It is generally agreed that secretin is an ampholyte, although there is a divergence of opinion as to the nature of the acidic and basic groups present. Mellanby [1928] reported that his concentrate did not contain any free amino-groups; it was precipitated by acids, which he ascribed to the high content of

[1] The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

organic phosphorus. Nevertheless, he produced evidence that indicated that secretin formed salts with both acids and metallic bases.

The experiments described in this paper support the view that secretin is a secondary proteose.

Experimental.

Preparation of an active concentrate.

1. Since Mortimer and Ivy [1929] had reported that the method of Mellanby [1928] had failed in their hands, the "new secretin" of Weaver, Luckhardt and Koch [1926] was used as the starting material for purification.

The fresh intestine of a dog, excised within twenty minutes of death, was washed through with tap-water, filled with about 250 cc. of 0·4 % hydrochloric acid, and the ends were clamped. After half an hour at room temperature, the liquid was run out, strained through muslin, and the filtrate saturated with sodium chloride. The extraction was repeated, the two extracts being combined. The bulky precipitate produced by saturation with sodium chloride had the properties of the "new secretin" described by Weaver, Luckhardt and Koch. It was usually white, but sometimes coloured yellow by a trace of bile. Successive extracts were stored in the refrigerator until a large batch of crude material had been obtained. It was found that the activity did not deteriorate in the cold.

2. *Deproteinisation.* The precipitate was separated from the solution by centrifuging. The supernatant liquid, which contained most of the depressor substance, was poured off and rejected. The precipitate was mixed with enough 0·4 % hydrochloric acid to form a thin cream, and heated to boiling in a large dish. While boiling, the mixture was brought with 2*N* sodium hydroxide solution to approximately p_H 4·0 in accordance with the original method of Bayliss and Starling [1902] for preparing secretin, and then to approximately p_H 6·0 with 5 % sodium acetate solution. The solution was filtered hot through muslin and filter-paper and allowed to cool. The precipitate, which consisted of coagulated protein, was rejected, the activity having passed into the filtrate.

3. *Precipitation with trichloroacetic acid.* Solid trichloroacetic acid was added to the filtrate from step 2 to make a 5 % solution as described by Still [1930]. A white turbidity was produced, and after the mixture had stood for an hour in the refrigerator the precipitate was found to have flocculated. It was centrifuged off, washed with aldehyde-free acetone and ether, and dried. It contained all the secretin, together with some protein, but was free from depressor substance and bile pigments. It was found to retain its activity for periods of three months or more when stored in the refrigerator, and was therefore used as a standard for comparison with new products.

4. *Extraction with alcohol.* The precipitate from step 3 was extracted with alcohol under a variety of conditions in order to find the maximum concentration of the alcohol and the optimum temperature to ensure that the

secretin went into solution. The 90 % acid alcohol solution advocated by Still [1930] failed to dissolve the secretin at room temperature, and was somewhat unreliable at 40°. The solution finally adopted was 85 % neutral alcohol (1 cc. for every 10 mg. of solid product), which dissolved the secretin nearly completely; further dilution resulted in the passing into solution of some of the protein impurities of the trichloroacetic acid precipitate. Extraction was carried out for an hour at 40–50°, which was found to be the optimum temperature; at lower temperatures solution did not take place very readily, while above 60° there was a risk of destruction of secretin by the alcohol. At the conclusion of the extraction, the insoluble residues, consisting chiefly of proteins, were centrifuged off. The removal of impurities with brucine and pyridine as recommended by Still [1930] was tried, but resulted in a serious loss of activity. The solution was therefore treated immediately after centrifuging with one volume of aldehyde-free acetone and five volumes of ether as in Still's procedure, and kept for an hour in the refrigerator. The precipitate, which contained the secretin, was then centrifuged off, washed with acetone and ether, and dried. This product was free from insoluble protein, but contained some sodium chloride.

5. *Precipitation as picrate.* This step was adapted from the method used by Dudley and Starling [1924] for the purification of insulin. The precipitate from step 4 was dissolved in distilled water to make a 1·5 % solution, and a trace of insoluble material centrifuged off. The clear, faintly yellow solution was treated with half its volume of saturated picric acid solution and stirred thoroughly. The preparation was stored in the refrigerator for twenty-four hours, by which time the supernatant liquid had become almost clear. It was then decanted from the precipitate, and the small portion of the precipitate still suspended in it was centrifuged down. The clear solution, on addition of saturated picric acid solution, gave no further precipitate.

The precipitate in the beaker was rubbed up with a quantity of a dilute solution of picric acid—5 cc. of saturated solution in 100 cc.—and centrifuged. The washing of the precipitate with dilute picric acid was repeated twice to free the picrate completely from sodium chloride; the use of the dilute picric acid minimised the risk of secretin passing into solution as was observed by Takács [1928, 1]. The washed picrate was dissolved in a volume of alcoholic HCl solution—1 cc. 3*N* HCl to 3 cc. absolute alcohol—approximately equal to the original volume of saturated picric acid used. The precipitate in contact with the acid formed brown, sticky drops, which were triturated in the solution until they were decolorised. The small quantity of insoluble material was centrifuged off and discarded. The solution was then diluted with ten volumes of aldehyde-free acetone. The white, flocculent precipitate was centrifuged off, washed twice in the centrifuge-cup with acetone and twice with ether, and dried over sulphuric acid *in vacuo*.

The secretin concentrate prepared by this method was free from depressor substance, insoluble proteins, sodium chloride and picric acid.

Physiological assay.

Cats were used for the experiments. The animals were anaesthetised by an intraperitoneal injection of urethane (1·0–1·25 g. per kg. body-weight).

Pancreatic flow was observed by noting the movement of a small bubble in a graduated 1 cc. pipette, clamped horizontally, filled with normal saline, and connected to the pancreatic cannula. For a few of the earlier experiments, an electric drop-recorder was attached to the open end of the pipette, but the use of this was found to be impracticable, since the protein present in the secreted juice was coagulated by the heat generated by the heavy current required to operate the signal, and fouled the platinum contacts. Throughout half of this work, the pipette alone was used for measurement of flow. For the latter half of the work there was added a thermionic drop-recorder devised by Dr D. T. Harris, the use of which was not hampered by the coagulation difficulty. The contacts were included in the grid circuit, where only a few amperes were necessary, while the signal was placed in the plate circuit, where there was ample power to operate it. The mechanism of this recorder has already been described [Harris, D. T., 1931].

While the pipette alone was in use, preparations were tested with a view to determining the minimum quantity of solid material necessary to evoke any secretion, which was defined as the threshold dose. After the introduction of the thermionic drop-recorder, the unit adopted was the "threshold dose" of Ivy *et al.* [1930], who define it as "that amount of dried material in solution which, when injected intravenously, will cause a 10 drop (0·4 cc.) increase in the rate of flow of pancreatic juice within a ten-minute period following the time of injection, as compared with the preceding ten-minute period."

The products from the various stages of purification were each assayed a large number of times. The activity was found to vary with the cat, the same preparation sometimes evoking secretion of twice or three times as much juice with one animal as with another. With the cruder products, the activities of different batches also varied.

The figures below give approximate values of the threshold doses for different stages of purification.

	Threshold dose (after Ivy)
Precipitate from step 3	8 mg. (approx.)
Precipitate from step 4	2 mg. (approx.)
Final product	0·5 mg.

The final product of Still [1930] was active in doses of 0·2–1·0 mg. per kg. in a 10 kg. dog, while Ivy *et al.* stated that the average threshold dose of their concentrate was 0·6–0·7 mg. These workers used methods of extraction closely related to the present method, which yields a product of activity comparable with those of their products.

The acid extraction method, however, was unsatisfactory in many respects compared with Mellanby's method, as was shown by the assay, with the above

technique, of a sample of Mellanby's own preparation, kindly supplied by him. The Ivy threshold dose for this preparation was found to be 0·05–0·1 mg.

Chemical properties of the concentrate.

The final product—"hydrochloride"—was a fine white powder, dissolving slowly in water to form a colourless solution. It was soluble over the whole range of p_H, but normally its aqueous solution had a p_H of 3·0 or less, whereas Mellanby's product in solution had a p_H of 8·0.

The preparation gave positive tests for the biuret (pink colour), xantho-proteic, Hopkins-Cole, Millon, Pauly (histidine colour) and sulphur reactions. The ninhydrin and Molisch reactions were negative.

A trace of precipitate was produced by half saturation of the aqueous solution with ammonium sulphate, while complete saturation produced a large precipitate.

Precipitates were produced by picric, tannic, and phosphotungstic acids, but not by sulphosalicylic or trichloroacetic acid.

The aqueous solution produced no coagulation when heated, and did not reduce Benedict's solution.

The activity was destroyed by boiling with 2*N* acid or alkali, and also by hot methyl or ethyl alcohol.

These results suggest that the secretin molecule is about the size of that of a peptone, and contains no carbohydrate or other reducing group. The sulphur must therefore be present in the disulphide form.

When kept in the refrigerator, the protein-free products generally retained their activity for long periods. There were, however, a few cases where the dried precipitate from step 4 of the purification process deteriorated somewhat rapidly even in cold storage. The cause of this has not been ascertained. In connection with this stability question, it may be mentioned that two active samples of Ivy's product, supplied by him to Dr Brocklehurst, were found to deteriorate slowly when kept at room temperature.

Behaviour of secretin with precipitated proteins.

In the early stages of the working-out of the above method of purification, attempts were made to extract the secretin from the sodium chloride precipitate of step 1, which contained both proteins and the depressor substance, by the method of Weaver [1927], which consisted in redissolving the precipitate as far as possible in distilled water, filtering off the undissolved material and saturating the filtrate again with sodium chloride. For this purpose the precipitate was suspended for an hour in a volume of distilled water about three-quarters that of the original acid solution. At the end of this time the insoluble slimy residues were filtered off, the filtrate was saturated with sodium chloride, and kept overnight in the refrigerator. The flocculent precipitate was centrifuged, washed with acetone and ether, and dried in air. Assay showed that this

product still contained a large quantity of protein, which resulted in a low activity.

The isoelectric precipitation procedure used by Ivy *et al.* [1929] for the purification of cholecystokinin was added to this method in order to remove proteins. The solution in distilled water, after the insoluble proteins had been filtered off, was adjusted to p_H 5·0–6·0 with $N/10$ sodium hydroxide solution. A white precipitate appeared, and was centrifuged off and discarded. Saturation of the solution with sodium chloride, or addition of trichloroacetic acid, then produced a second precipitate, which was centrifuged off, washed and dried as before.

Assay showed that products prepared by this method were either inactive or considerably less active than those prepared by the same method with the isoelectric precipitation step omitted. A considerable quantity, but not all, of the protein present had been removed by the isoelectric precipitation. This result suggested that the precipitated protein had adsorbed all or part of the secretin, and so removed it from solution.

Further evidence in confirmation of this view was obtained from examination of the slimy protein material that had failed to dissolve when the sodium chloride precipitate from the intestinal extract was suspended in distilled water. This material was extracted by the Bayliss-Starling procedure as in step 2 of the final method of preparation, and the extract evaporated down to about 25 cc. in a current of air at 40°. This solution when injected was found to be very highly active, 0·5 cc. evoking a steady flow lasting twenty minutes. This showed that very little of the secretin was dissolved by suspending the crude material in distilled water, even though it contained a large quantity of 0·4 % hydrochloric acid, the greater part of the active material remaining adsorbed on the protein.

In the subsequent steps of the purification process, some further observations were made on the behaviour of secretin with precipitated protein. In step 3, the active material was precipitated from solution with trichloroacetic acid, the resultant precipitate still containing a considerable quantity of protein. The aqueous solutions of the precipitate from step 4 and the final product, however, which were protein-free, produced no precipitates with trichloroacetic acid; the success of step 3 therefore was dependent on the presence in the solution of protein, which was precipitated by the trichloroacetic acid, and carried the secretin down with it by adsorption. Step 1 also depended on protein adsorption. In connection with this, it may be mentioned that Necheles and Lim [1927], having recovered a pancreatic secretory excitant from the circulating blood of a dog by vivi-dialysis, expressed doubts as to whether this was secretin, since it could not be precipitated from solution by saturation with sodium chloride. Since dialysis was carried out through goldbeater's skin membranes into Locke's solution, their dialysate could not have contained any protein, so that precipitation with sodium chloride was impossible.

Observations were also made on the limitations of the adsorbing power of precipitated proteins. It was evident from step 2 that coagulation of the

proteins by heat also destroyed their adsorbing power to a great extent. (A small quantity of secretin could be recovered by extracting the coagulated protein with alcohol.) The dried precipitate from step 3, when required for assay, was dissolved in distilled water or normal saline, and the solution brought to p_H 6·0–7·0. The protein thus precipitated could be filtered off without loss of activity, the secretin remaining in solution. This suggests that the acetone and ether that were used to wash the precipitate had a denaturing effect on the protein, which destroyed its adsorbing power; it was a more profound change than mere dehydration, since it could not be reversed by water.

Adsorption of secretin on benzoic acid.

Mellanby [1928] attempted to absorb secretin on benzoic acid, but was unsuccessful, since his product was precipitated from solution by acids. Since in the present research there had been no evidence of precipitation by acids, the behaviour of the present product with benzoic acid was investigated with a view to its possible application to the purification of secretin.

The solution from step 2 was used for the first experiments. It was mixed with about a quarter of its volume of a 10 % solution of commercial sodium benzoate, and the mixture acidified by slow addition of 2*N* hydrochloric acid until the benzoic acid was completely precipitated. This was then filtered off on a hardened filter, washed with water, and pressed as dry as possible. It was then spread in thin layers on glass plates, which were stacked in a vacuum desiccator, and dried for two or three days over calcium chloride and phosphorus pentoxide. When dry, the benzoic acid was rubbed off the plates, powdered in a dry mortar, and dropped into a quantity of ether that had been dried over sodium. The benzoic acid dissolved, leaving a few mg. of a semi-transparent solid of a light brown colour, which was filtered off on a hardened filter, washed with the dried ether, collected and assayed.

Results of the physiological assay of the adsorbate and of the filtrate from the benzoic acid precipitation showed that the whole of the activity had been adsorbed from the solution by the benzoic acid. The adsorbates were all free from the depressor substances, which had remained in the filtrates. The adsorbate still contained a small quantity of protein, and was also contaminated with some black specks that were considered at the time to have come from the commercial sodium benzoate. The evidence suggested that the method would give more promise when applied to a protein-free product.

The precipitate from step 4, which was protein-free, was later subjected to similar tests. For these experiments the commercial sodium benzoate was replaced by benzoic acid, which was recrystallised from hot water before use. It was then dissolved in excess of 2*N* sodium hydroxide, and the solution filtered. The secretin preparation, dissolved in distilled water, was added to the benzoate solution, the mixture stirred for a second, and the benzoic acid then precipitated as rapidly as possible by addition of concentrated hydrochloric acid with constant stirring. From this point, the procedure was the same as in

the earlier experiments, except that the adsorbate was centrifuged from the ether solution, since hardened filters were found to adsorb secretin [*cf.* Mellanby, 1928].

Assay of this product showed beyond doubt that secretin was adsorbed by benzoic acid. The adsorbate in general was found to have double the activity of the original precipitate from step 4. It was, however, always contaminated with the insoluble black specks that had been noticed in the earlier experiments, however carefully the benzoic acid had been purified. These specks settled very readily from the aqueous solution and could be centrifuged off.

The great drawback to the application of this process to the purification of secretin was its very small yields. From 50 mg. of the original material only $\frac{1}{8}$ of its weight of adsorbate was obtained, yet the activity was only doubled. This suggested that only a limited quantity of secretin could be adsorbed by a given weight of benzoic acid. An attempt was made to avoid loss due to this cause by using excess of benzoic acid, but the adsorbate was found to be contaminated with a large quantity of sodium chloride, which was probably occluded by the benzoic acid on precipitation.

Ultrafiltration.

Lalou [1912] and Takács [1928, 2] both succeeded in obtaining ultrafiltrates of secretin through thick collodion membranes, but over 50 % of the activity was lost. Their ultrafiltrates, however, still showed a measurable activity, especially that of Takács, which he showed by the sulphosalicylic acid test to be protein-free. This indicated that the secretin molecule was not as large as that of a protein. Further ultrafiltration experiments were carried out in the present research to obtain more definite evidence as to the size of the molecule.

Ultrafiltration through cellophane. The experiments were carried out in a Bechhold ultrafilter. A disc of cellophane, supported by a disc of 60 mesh silver gauze, was placed between two well-greased rubber rings, and introduced, with the cellophone uppermost, into the ultrafiltration chamber. The perforated plate at the bottom of the ultrafiltration chamber, on which the membrane rested, was plated with silver to avoid the possibility of metal being dissolved by hydrochloric acid.

The cellophane was first tested with 1 % solutions of egg-albumin and peptone. In both cases liquid passed through the membranes at the rate of one drop per minute. The ultrafiltrate from the albumin solution contained no protein as proved by the sulphosalicylic acid test, while that from the peptone solution gave precipitates with both tannic and phosphotungstic acids, showing that the cellophane was permeable to peptone but not to albumin.

A strong solution of the precipitate from step 4 (40 mg. in 20 cc. water) was then ultrafiltered through cellophane. On application of a pressure of 3–4 kg. per cm.^{2}, the solution passed through the membrane at the rate of one drop per minute, no visible residue being left above the membrane. The ultrafiltrate was assayed, together with the original solution.

In three experiments out of four, the ultrafiltrate was found to be active, the activity having been reduced to about $\frac{1}{6}$ of that of the original solution. It was observed that the fourth ultrafiltrate, which was inactive, was the only one of this series that gave a negative biuret reaction.

It can be concluded from these experiments that secretin has a larger molecule than a peptone, and can only pass through the largest pores of a cellophane membrane.

Collodion membranes. A series of graded collodion membranes were made by the method of Desai [1930].

The stock collodion solution was a 2 % solution in anhydrous alcohol and ether in the proportion of 1 : 3, obtained from British Drug Houses, Ltd. From this stock solutions were prepared containing 1, 2, 4 and 8 % by volume of ethylene glycol as swelling agent. Sets of membranes of four grades of permeability were then made from these four solutions. Desai had found that with the aid of membranes prepared from these solutions, peptones, secondary proteoses, and primary proteoses could be separated from one another by dialysis. Since the precipitation tests had been found to support the assumption of Mellanby and Huggett [1926] and others that secretin was a secondary proteose, it was considered that this range of membranes should serve for the identification of the secretin molecule.

The nitrocellulose/water ratios of the membranes were determined by the method described by Pierce [1927], several determinations being made for each grade of membrane, and the mean value taken as the correct ratio.

Collodion membranes.

Membrane series	% Ethylene glycol in solution	Nitrocellulose/ water ratio	Desai's value
I	1	1 : 0·5323	1 : 0·510
II	2	1 : 1·4048	1 : 1·050
III	4	1 : 2·2852	1 : 2·083
IV	8	1 : 2·7640	1 : 4·102

Series IV was not prepared until several months after the other three series, the stock collodion solution meanwhile having been stored in the refrigerator. This may account for the high nitrocellulose/water ratio, compared with that of the corresponding membrane of Desai's series.

The membranes were first tested with albumin and peptone solutions. Both solutions filtered very rapidly through a membrane of series IV under a pressure of 2·25 kg. per cm.^{2} The membrane was found to allow the passage of peptone, but to retain albumin. With membranes of series III, ultrafiltration proceeded at a similar rate, but all but a trace of the peptone was retained. Membranes of series I were found to be impermeable to all aqueous solutions, even under a pressure of 5 kg. per cm^2.

The membranes of series III were tested with solutions of the secretin concentrate in normal saline (20 mg. in 10 cc.). In each case the solution

passed through the membrane very rapidly, leaving no visible residue. The ultrafiltrates, however, were all found to be inactive, showing that membranes of series III were impermeable to secretin. Membranes of series IV were then tested with similar solutions, the whole solution passing through the membrane on each occasion, as in the case of series III. These ultrafiltrates were also found to be inactive, showing that the secretin molecule is larger than that of a peptone, a conclusion borne out by the absence of any precipitate when most of the ultrafiltrates were tested with picric acid. On one occasion, however, the ultrafiltrate from one of this series of membranes was found to give a precipitate with picric acid; unfortunately, the assay of this particular ultrafiltrate was prevented by an accident. This result suggests that the permeability of the average membrane of series IV is just below the minimum permeability required to permit the passage of molecules of the size of secretin. A membrane of nitrocellulose/water ratio about 1 : 4 would probably be permeable to such molecules. Desai has shown that a membrane of this ratio allows the passage of all secondary proteoses, but not of primary proteoses; this points to secretin being a large secondary proteose, as first suggested by Mellanby and Huggett [1926].

Discussion.

The adsorption of secretin from acid and neutral solutions by precipitated protein is an important factor to be considered in the purification of the hormone, and in the determination of its chemical nature.

Since the adsorbing power of native proteins is destroyed by their denaturation with organic solvents, loss of secretin by adsorption is reduced to a minimum by the denaturation of the protein as soon as possible after precipitation with sodium chloride. In the present research, the deproteinisation of the precipitate by the Bayliss-Starling method, and the subsequent precipitation with trichloroacetic acid were carried out before denaturation because these two steps resulted in a complete separation of secretin from the basic depressor substance, which remained in the acid solution.

Mellanby extracts the duodenal mucosa directly at room temperature with absolute alcohol, which destroys the adsorbing power of the proteins present. If the alcohol is sufficiently dilute to dissolve some of the protein, however, the adsorbing power of this dissolved protein is not destroyed; this is shown in the method of purification worked out by Ivy *et al.* [1930], in which the secretin was extracted from the crude sodium chloride precipitate of Weaver, Luckhardt and Koch [1926] with 75 % alcohol at room temperature. Since the active material in the resultant alcoholic extract could be precipitated with trichloroacetic acid after the removal of the alcohol, it follows that some of the proteins had passed into solution without undergoing loss of adsorbing power.

Ivy and his co-workers, by isoelectric precipitation at p_H 5·3–5·7, separated their secretin concentrate into two fractions, one of which was soluble in water, and the other only in 0·4 % hydrochloric acid, and which they suggested were

two different secretins. Since both these fractions were precipitated from solution by trichloroacetic acid, it follows that neither was protein-free; they were probably adsorbates of secretin on proteins of different isoelectric points. No other evidence has been obtained suggesting the existence of more than one secretin.

Mellanby's observation that secretin was precipitated from solution by acids was not confirmed, the product of the present research remaining in solution over the whole p_H range on the acid side of neutrality. Since, however, its potency was only about 1/10 of that of Mellanby's product, this may have been due merely to the small quantity present.

The adsorption of secretin by precipitated protein in acid and neutral solutions and by benzoic acid is in close agreement with the other cases of adsorption already stated by Mellanby, who found that in neutral solution adsorption took place on alumina and charcoal, but in alkaline solution the amount adsorbed diminished with rise of p_H. The evidence shows that adsorption takes place in acid or neutral solutions but is altered by addition of alkali.

The results obtained from the ultrafiltration experiments and precipitation tests suggest that secretin is a large secondary proteose rather than a polypeptide as suggested by Mellanby. Further evidence of its peptide structure is afforded by its rapid destruction by proteolytic enzymes, and by indications of salt formation observed by Mellanby [1928]. The active part of the molecule with respect to adsorption should therefore be the positive ion, for in accordance with the zwitterion constitution of amino-acids and proteins [see Harris, L. J., 1930], this ion retains its charge in acid and neutral solutions, but not in alkaline solution. The nature of this positive ion is an open question, for Mellanby has shown that the molecule contains no free amino-groups.

Summary.

1. A method is described for preparing a secretin concentrate, free from depressor substances, insoluble proteins, and inorganic salts.

2. The threshold dose of this concentrate is about 0·5 mg. for a cat of 2·5 kg. body-weight.

3. Chemical and precipitation tests indicate that secretin is a secondary proteose.

4. Secretin is soluble in water over the whole range of p_H.

5. Secretin is adsorbed readily by precipitated proteins from acid and neutral solutions. The adsorbing power of the proteins is destroyed by denaturation with organic solvents.

6. Secretin can be adsorbed from solution on benzoic acid.

7. Secretin can be ultrafiltered through cellophane. It is retained by a collodion membrane permeable to peptones.

8. The above results are discussed fully and compared with those of other workers. It is suggested that the adsorption of secretin is associated with its positive ion.

I am greatly indebted to Professor J. C. Drummond for his kind advice and helpful criticism throughout the whole of this work, to Dr R. J. Brocklehurst and Dr D. T. Harris for the physiological assay of the various products, to Dr Harris also for devising his thermionic drop-recorder, and to Prof. J. Mellanby for a sample of his highly active secretin concentrate for use as a standard.

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CXXVII. A BIOLOGICAL SYNTHESIS OF *m*-DIGALLIC ACID.

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WHATEVER position is assigned to the glucose residue it is now generally agreed that gallotannin is mainly a derivative of *m*-digallic acid [Schiff, 1871, 1872; Nierenstein, 1905, 1910; Fischer and Freudenberg, 1912, 1913, 1914; Fischer, Bergmann and Lipschitz, 1918; Herzig, 1923; Nierenstein, Spiers and Hatcher, 1925], which is thus the source from which gallic acid is produced on hydrolysis. In the case of tannase hydrolysis it was to be expected that the reaction would be reversible and this is now confirmed, since both *A. niger* and tannase produce *m*-digallic acid to a small extent from gallic acid. Gallotannin is of pathological origin, probably produced from gallic acid by the gall-forming larva, which by its tannase content [Nierenstein, 1930] hydrolyses and also synthesises gallotannin. This probably accounts for the close admixture of these two components and for the fact that so far no complete separation of gallotannin from gallic acid has been successfully carried out, a point investigated by other workers in this laboratory [Hooper, 1925; Nicholson and Rhind, 1924].

EXPERIMENTAL.

I. *General.* The cultivation of *A. niger* in gallic acid solution was carried out as already described and this also applies to the preparation of the tannase-pyrogallase mixture [Nicholson, Nierenstein, Pool and Price, 1931].

II. *Identification of* m-*digallic acid.* The solutions of gallic acid which had been acted on by *A. niger* or tannase were extracted with ether and the residue left on evaporation of the ether was crystallised from a large quantity of water (about 500 cc. for 2 g. of residue). Each fraction thus obtained was collected, dried and methylated with diazomethane in ethereal solution. Most fractions proved to be trimethylgallic acid methyl ester on crystallisation from methyl alcohol, but a small quantity (about 0·3 g. from 2 g. residue) was found to melt correctly (M.P. 127–128°, mixed M.P. with an authentic specimen 127–128°) for pentamethyl-*m*-digallic acid methyl ester after 3 to 5 crystallisations from methyl alcohol. ($C_{20}H_{22}O_9$ requires C = 59·1; H = 5·4. Found: C = 58·9; H = 5·5.)

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CXXVIII. THE PENTOSAN CONTENT OF *KLEINIA ARTICULATA.*

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The results of this paper represent part of a preliminary survey of the metabolism of the succulent composite *Kleinia* (*Senecio*) *articulata.*

The formation of mucilaginous substances of a pentosan nature has been put forward as a direct cause of the succulent habit [McDougal *et al.*, 1918, 1919]. It is claimed that the peculiar metabolism of succulents involves a partial change from a hexose to a pentose metabolism, this change being facilitated by exposure to high temperatures and to a low water supply. This view has been discussed in a previous publication [Evans, 1932].

The sap of *K. articulata* after filtration did not appear to be at all mucilaginous, and some quantitative knowledge of the pentosan content seemed desirable.

Some investigators [Rosa, 1921; Doyle and Clinch, 1926] have distinguished between water-soluble pentosans; those insoluble in water but hydrolysed and brought into solution by 1 % HCl; and those insoluble in water but which require fairly concentrated (12 %) HCl to hydrolyse them and bring them into solution.

The exact nature of these three classes of pentosans and the part which they play in the metabolism or structure of the cell is unknown. Nevertheless it is fairly probable that the pentosans extractable by hot water alone are normally in solution (either true or colloidal) in the cell-sap, and that those which are only hydrolysed by 12 % HCl represent part of the wall structure.

It must be remembered however that the pentosans are not the only substances which contain pentose sugars in their molecule; in particular, hemicelluloses and pectic substances also generally contain pentoses, which are set free on hydrolysis. According to Onslow [1931], a certain class of compounds, of which glycuronic and galacturonic acids are members, produce furfuraldehyde under the same conditions as pentoses and pentosans. In most of the pentosan determinations such substances are also included, and the determinations thus approximate more closely to a measure of pentose metabolism than to a measure of any particular class of pentose-containing substances.

Those pentosans which are extracted by 1 % HCl, and especially by 12 % HCl, are probably part of the wall-structure, and possibly include pectic

substances and other complex polysaccharides brought into solution by the acid.

The pectic substances in particular have been shown to be of more or less universal occurrence in plants [Carré, 1929]. According to Onslow [1931] they are found to the greatest extent in fleshy and succulent tissues.

It is evidently those pentosans which are in solution in the cell-sap, and which presumably form the bulk of the pentosans extractable by water, which are of greatest interest in connection with the part played by pentosans in the causation of succulency.

Nevertheless the pentosan theory of succulency has been based upon the total pentosans (and other pentose-containing substances) extractable by 1 % HCl. No data are apparently available regarding the water-soluble pentosan content of succulents.

In the present work the three classes of pentosans, as separated by solubility in water, 1 % HCl and 12 % HCl respectively, have been determined wherever possible.

Method.

About 12 similar plants were taken for the determinations, their fresh weight being determined, and they were then cut into small portions about 0·5–1 cm. long. These small pieces were well mixed, and about 10 g. used for a dry weight determination.

The remainder were immediately killed by plunging into boiling alcohol containing a little strong ammonia, and boiled for about 30 minutes. They were then separated from the alcohol, placed in an oven at 95° and thoroughly dried. The alcohol belonging to each set was carefully kept.

The dried plants were finely ground in a mortar and transferred into a strong round-bottomed flask fitted with a reflux condenser, together with the alcohol that had been used for killing them.

The mixture was boiled gently for about 5 hours, by which time the alcohol-soluble sugars were entirely removed; the alcohol was filtered off, and the residue washed repeatedly with fresh portions of alcohol, which were added to the main bulk.

This procedure not only prevents any hydrolytic and enzymic changes in the tissue by almost instantaneous killing, but the preliminary drying and grinding of the tissues greatly shorten the time required for complete extraction of the sugars. The alcohol-soluble sugars were estimated in a few cases, but attention was paid in the main to the sugar-free residue.

This was dried and boiled with water for 5 hours. The residue was filtered off while hot and the watery extract kept for determinations of water-soluble pentosans. To the residue were added 150–200 cc. of 1 % HCl, and digestion was continued at the boiling-point for 3 hours. The mixture was filtered and the filtrate kept for the determination of the 1 % HCl-soluble fraction. The residue was retained for the determination of "residual" or 12 % HCl-soluble pentosans.

The pentosan contents of the three fractions were determined by distillation with 12 % HCl under Kröber and Rimbach's conditions [1902], the pentosan being thus converted into furfuraldehyde which was precipitated as phloroglucide in the distillate. All the determinations were carried out under similar conditions so that the results are comparable.

Results.

In order to obtain some estimate of the degree of uniformity to be expected in the plants used, three samples were taken at the same time, each sample consisting of five similar plants.

The results of these determinations are given in Table I.

Table I. *Mature stems taken 12.30 p.m., October 30th.*

No of set	Dry matter %	% of dry weight			
		Water-sol.	1 % HCl-sol.	12 % HCl-sol.	Total
1	3·73	0·75	4·44	2·82	8·01
2	4·00	1·25	4·58	2·39	8·22
3	3·53	0·88	4·59	2·48	7·95

The total pentosan contents of these samples agreed fairly closely. Regarding the slight differences, it is interesting to note the inverse relation between total pentosan content and water content, though this is of doubtful significance.

The proportions of pentosans in the three fractions also differ slightly, but the main outcome of the experiment is to show that only about one-eighth of the total pentosan is in the water-soluble form.

The results of further pentosan determinations are given in Table II. Each sample of these experiments contained 12–15 similar plants. In general there is no great difference between the water-soluble pentosan content of leaves and young stems, and of more mature shoots, the slight difference that was shown in most of the experiments indicating a slightly higher amount in the former.

The total pentosan content of leaves however is much less than that of stems, the difference becoming more marked with increasing age of the stem. In the case of both stems and leaves the greater part of the pentosans is that fraction which is hydrolysable by 1 % HCl. In old joints however, as would be expected, the proportion of the residual or 12 % HCl-soluble pentosans is considerably higher.

In the case of stems the quantity of 1 % HCl-soluble pentosans increases considerably with age up to maturity, but decreases in very old stems. Most of the material denoted as "old" in the experiments of Table II had not passed the mature stage, but that of Exp. 3 was definitely older.

Table II.

Description of material	Part of plant	% of dry weight: Dry matter %	Water-sol.	1 % HCl-sol.	Residual or 12 % HCl-sol.	Total pento-san	Total "loose" *i.e.* 1 % HCl-sol. + water sol.
Exp. 1. *Kleinia articulata*. Outer house. Sept. 11th, 1930	Whole plants	—	—	—	—	—	—
Exp. 2. *K. articulata*. Similar material to that of Exp. 1, but slightly younger. Sept. 12th, 1930	,,	6·46	—	—	1·94	7·34	5·40
Exp. 3. *K. articulata*. Old joints (1929 growth), young joints (1929–30 winter growth) bearing leaves. Inner house. Sept. 22nd, 1930	1. Old joints 2. Young joints 3. Leaves	5·72 4·27 4·30	0·664 0·757 0·740	2·34 5·44 —	3·64 2·60 —	6·64 8·80 —	3·00 6·20 —
Exp. 4. *K. articulata*. Long slender plants grown in shade from cuttings planted Nov. 20th, 1929, old joints 1929–30 growth, and young joints of 1930 summer growth bearing large leaves. Oct. 6th, 1930	1. Old joints 2. Young joints 3. Leaves	5·25 3·57 2·56	0·38 0·34 0·44	4·61 4·13 2·33	3·45 2·87 1·97	8·44 7·34 4·74	4·99 4·47 2·77
Exp. 5. *K. articulata*. Plants grown from cuttings made July 15th. From Sept. 18th onwards. I. Watered daily. II. Left without water. Analysed Oct. 18th, 1930	I. Young joints I. Old joints II. Young joints II. Old joints	3·54 5·64 5·68 8·00	— — — —	— — — —	2·02 4·07 1·96 3·61	6·17 9·77 6·76 8·41	4·15 5·70 4·80 4·80
Exp. 6. *K. articulata*. Plants from same box as material of Exp. 5, growing under normal conditions from July 15th to Oct. 30th. Oct. 30th, 1930	Young joints + leaves Old joints	3·85 5·23	— —	— —	2·12 3·56	5·67 9·94	3·55 6·38
Exp. 7. *Mesembryanthemum edule*. Leaves of one large plant used. Oct. 10th, 1930	Leaves	9·24	1·80	3·76	0·23	5·79	5·56
Exp. 8. *Fuchsia* leaves. Oct. 9th, 1930	Leaves	16·03	1·05	1·53	2·05	4·63	2·58

Plants which were grown in the shade (Exp. 4), which are thin and tender compared with normal plants and which have a relatively greater water content, have a very low pentosan content, this being especially so for the water-soluble fraction.

An experiment was carried out to test the effect of dryness on the pentosan content.

From a batch of similar plants two samples were transplanted. One of these samples was watered daily for one month, the other was kept unwatered, and both were analysed at the same time (Exp. 5). Another sample which had been left under ordinary conditions was analysed a fortnight later (Exp. 6).

The differences are irregular and do not reveal any appreciable effect of dry conditions on pentosan content. The differences between samples I and II in terms of fresh weight are to be interpreted largely as the reflection of a reduction in the fresh weight of the unwatered plants which was used as the basis for the calculations.

For comparison with *Kleinia*, pentosan determinations were carried out for the leaves of *Mesembryanthemum edule* and *Fuchsia*; the results are given in Table II (Exps. 7 and 8).

It is obvious that, although the total pentosan content of *Kleinia* may be greater than that of *Mesembryanthemum* and *Fuchsia*, the proportion in the water-soluble form is much higher in the latter plants.

While the water-soluble pentosan (expressed as a percentage of the total) is about 5–15 % in *Kleinia*, in *Mesembryanthemum edule* it is 32 %, and in *Fuchsia* 44 %. The absolute amounts of water-soluble pentosans are also higher in these plants than in *Kleinia*. In all these experiments large samples were used and the results represent a mean value for these particular plants.

It may be objected that the preliminary extraction of the tissues with alcohol to remove soluble sugars may cause a physical change in the water-soluble pentosans so that subsequently they are not easily soluble in water. This would mean that the pentosans extractable by hot water did not correspond in quantity to those actually in solution in the sap of the intact plant.

In order to test this point, an experiment was carried out, in which the fresh juice was expressed and pentosan determinations made on the extracted sap, and on the residual tissues.

	Pentosan % fresh weight	
	Sap	Residue
Mature plants of 1931 summer growth. Taken 11 a.m. 5. ii. 32	0·028	0·462

The pentosan in solution in the cell-sap corresponds closely to the quantities previously determined in the watery extract. Calculating data from Table II as percentages of the fresh weight, the water-soluble pentosan of stems ranges from 0·012 to 0·038 and the corresponding total pentosan from 0·262 to 0·443.

For *Mesembryanthemum* the figures in terms of fresh weight are 0·167 water-soluble and 0·536 total; for *Fuchsia* 0·169 and 0·744.

Summary and conclusions.

These determinations have shown that *Kleinia articulata* contains an amount of pentosan similar to that found in other succulents. It has also been shown however that only a small proportion of this is in solution in the cell-sap.

The major part of the pentosan comes within the fraction which is extracted by 1 % HCl, while there is also a considerable amount which is only extracted by 12 % HCl. It is probable that the greater part of this pentosan is derived from the breakdown of the cell-wall, and to some extent of the plant protoplasm.

As the water-soluble pentosans seem to be of the greatest interest in relation to the pentosan theory of succulence, the fact that *Kleinia* contains such a small amount does not lend much support to the theory, especially when it is considered that a plant like *Fuchsia*, which is not usually classed as succulent, contains greater quantities.

It is possible that the loosely bound pentosans extractable by 1 % HCl, in spite of their insolubility in water, may play a part in the water relations

of the cell, but it appears unlikely that a slightly greater quantity of these than that found in mesophytes is sufficient to transform the make-up of the plant, and to furnish it with the massive water-storage tissue characteristic of succulents.

I am indebted to Prof. D. Thoday for suggesting this work and for continued interest throughout.

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CXXIX. THE BASIC AMINO-ACIDS OF CRYSTALLINE EGG-ALBUMIN[1].

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The extensive investigations of Sørensen [1917, 1930] have provided evidence that crystalline egg-albumin conforms more closely than almost any other protein to the criteria for a homogeneous and reproducible chemical substance. He has also shown that its solubility in aqueous solutions of neutral salts follows the laws of physical chemistry with considerable precision, and that its osmotic pressure in solution is that of a substance of molecular weight close to 34,000, a magnitude that is supported by the results of Cohn, Hendry and Prentiss [1925], and of Svedberg and Nichols [1926]. Egg-albumin is therefore an eminently suitable substance to employ in studies of proteins as electrolytes in solution; its usefulness for this purpose has been restricted, however, by the inaccuracy and incompleteness of our present knowledge of its amino-acid composition.

There is good reason to believe that the acid- and base-binding capacities of proteins can be quantitatively accounted for in terms of the proportions of trivalent amino-acids (basic and dicarboxylic amino-acids) which they yield on complete acid hydrolysis [Cohn, 1925, 1931]; accurate determinations of the tervalent amino-acids are consequently of the first importance in studies of the electrochemical behaviour of proteins. We have therefore undertaken a determination of the basic amino-acids yielded by egg-albumin and, in order to facilitate the application of our results, have employed a standard preparation of the protein upon which electrometric titrations are being conducted in another laboratory.

At the time when the present analysis was begun no determinations by modern methods of the three basic amino-acids of crystalline egg-albumin had been made. Osborne, Jones and Leavenworth [1909] employed the procedure of Kossel and Patten [1903] (see Table I) and Skraup and Hummelberger [1909], who worked with commercial egg-white, used the original method of Kossel and Kutscher [1900]. Since then a few determinations of single bases only have been recorded and these are also shown in Table I.

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D.C.

Table I. *The basic amino-acids yielded by egg-albumin.*

Analyst	Albumin prepared by	Histidine %	Arginine %	Lysine %
Authors (1st analysis)	Cannan and Shore	1·43	5·30	4·97
Authors (2nd analysis)	"	1·25	5·31	4·69
Authors (3rd analysis)	du Vigneaud	1·48	5·56	4·78
Calvery [1932] (corrected)	Calvery	1·39	5·04	4·00
"	"	1·41	5·01	3·91
Calvery	du Vigneaud	1·38	5·11	3·78
Osborne, Jones and Leavenworth [1909]	Leavenworth	1·71	4·91	3·76
Skraup and Hummelberger [1909]	Commercial	1·5	2·9	3·9
Hanke [1925] (colorimetric)	—	2·3	—	—
Sakaguchi [1925] (colorimetric)	—	—	6·07	—
Fürth and Deutschberger [1927] (flavianate)	—	—	6·0	—
Hunter and Dauphinee [1930] (arginase)	Merck	—	5·02*	—

* This figure is calculated from Hunter and Dauphinee's data on the assumption that their egg-albumin contained 15·62% protein-N [Sørensen and Høyrup, 1917].

Just as we had completed the first analysis given in the table we learned, through a personal communication, that Dr H. O. Calvery of the University of Michigan had likewise conducted an analysis of crystalline egg-albumin. Dr Calvery kindly allowed us to see his results in advance of publication [Calvery, 1932]; his value of 5·03 % for arginine was in good agreement with our own, but his higher values of 2·44 % of histidine and 6·41 % of lysine induced us to repeat the analysis of our preparation of egg-albumin, and ultimately to analyse a preparation kindly furnished us by Dr du Vigneaud, of the University of Illinois, for this purpose[1]. The close agreement of the results of these three analyses was brought to Dr Calvery's attention and, on re-examining his own data, he found that an unfortunate error had been committed in his original calculations. The figures given in Table I represent the corrected results of Calvery's previous determinations of the bases of egg-albumin, and with them is given a new analysis of du Vigneaud's preparation carried out by Calvery simultaneously with our analysis of the same material.

Experimental.

First analysis. The albumin was prepared from fresh hen's eggs by one of the authors while working with Prof. R. K. Cannan at New York University and Bellevue Hospital Medical College. The method of Sørensen and Høyrup [1917] was used and the protein was crystallised three times from ammonium sulphate solutions at a reaction close to p_H 4·7. The product was dissolved in water, dialysed free from sulphate, and was then coagulated by being poured into a large volume of water heated to 70°. The coagulum was washed successively with liberal quantities of water, alcohol, and ether, and was then dried. It contained 15·95 % of nitrogen corrected for ash and moisture. The moisture content was 2·90 % and the ash 0·53 %. On distillation with mag-

[1] We are deeply indebted to Dr du Vigneaud for supplying us with this very fine preparation.

nesia the equivalent of 0·12 % of free ammonia-nitrogen was found; corrected for this the protein-nitrogen of the preparation was 15·83 %.

Of this material 191·4 g., corrected for moisture and ash, were boiled for 31 hours with 1600 cc. of 6*N* hydrochloric acid. The hydrolysate was concentrated *in vacuo* to a syrup which was diluted and again concentrated several times to remove as much of the acid as possible. The syrup was then diluted, sulphuric acid was added, and the remaining hydrochloric acid was removed by the careful addition of an excess of silver oxide, the reaction being maintained at p_H 2–3 with sulphuric acid. The silver chloride was thoroughly extracted with hot, very dilute hydrochloric acid in order to recover any histidine that may have been precipitated, and the extract was evaporated to dryness; the chloride remaining in this was removed in the same manner. The combined solutions were concentrated to 4 litres and an attempt was made to introduce an excess of silver ion by the alternate addition of sulphuric acid and silver oxide according to the technique of Kiesel [1926]. Although silver oxide was added until silver sulphate crystallised copiously from the solution, excess of silver ion could not be demonstrated by means of barium hydroxide. A similar behaviour has been repeatedly encountered in this laboratory and, apparently, the difficulty can be surmounted only by liberal dilution of the hydrolysate or by the use of silver nitrate; in the present analysis we chose the latter alternative. After the addition of excess of silver nitrate the precipitate of silver sulphate was removed and washed, and the arginine and histidine silver compounds were thrown down by adjusting the reaction to approximately p_H 12 with warm saturated barium hydroxide. From this point the analysis was conducted as described by Vickery and Block [1931]; the data from which the yields of the three bases were calculated are assembled in Tables II–IV.

Second analysis. The material used for the second analysis had been crystallised three times and was then dialysed and coagulated as before, washed and dried in the air. It contained 15·70 % of nitrogen calculated moisture- and ash-free. The moisture content was 12·22 % and the ash was 0·25 %. The amount taken was determined both by weighing the air-dry material and by analysis for nitrogen of an aliquot part of the hydrolysate, after this had been carefully agitated in order to bring any insoluble humin into suspension. The quantity carried on for analysis, as determined from the air-dry weight, was 72·35 g., as determined from the nitrogen in the hydrolysate, was 72·41 g.; the average of these, or 72·38 g., was employed in the calculations. Hydrolysis was effected by boiling the protein with 900 cc. of 6*N* hydrochloric acid for 25 hours; the acid was then removed as before. The filtrate from the silver chloride was brought to 5 litres and an excess of silver ion was introduced by means of silver oxide and sulphuric acid. Owing to the greater dilution of this solution with respect to amino-acids no difficulty was encountered. The procedure followed was that described by Vickery and Leavenworth [1928], except that the histidine fraction was freed from cystine

Table II. *Histidine fraction.*

Analysis	Protein taken for analysis g.	Vol. of histidine fraction cc.	Nitrogen content g.	Histidine equivalent in protein %	Vol. of fraction used for diflavianate cc.	Weight of diflavianate g.	Sulphur content of diflavianate. Theory 8·17 % %	Yield of histidine corrected %
1	191·4	500	0·7735	1·49	420	11·678	8·18	1·43
2	72·38	250	0·285	1·45	230	4·219	8·14	1·25
3	64·25	250	0·2887	1·66	230	4·434	8·17	1·48

Table III. *Arginine fraction.*

Analysis	Vol. of arginine fraction cc.	Nitrogen content g.	Arginine equivalent in protein %	Vol. of filtrates from arginine silver cc.	Solubility* correction for arginine silver 0·036 g. arginine per litre g.	Vol. of fraction used for flavianate cc.	Weight of flavianate g.	Arginine equivalent in 500 cc. of fraction g.	Sulphur content of flavianate. Theory 6·56 % %	Yield of arginine corrected %
1	500	3·474	5·64	12,500	0·450	50	2·717	9·687	6·61	5·30
2	500	1·532	6·58	9,200	0·331	100	1·9705	3·513	6·64	5·31
3	500	1·475	7·14	7,700	0·277	100	1·8480	3·294	6·52	5·56

* The weight of arginine calculated from the solubility correction is added to the weight derived from the flavianate.

Table IV. *Lysine fraction.*

Analysis	Vol. of lysine fraction cc.	Nitrogen content g.	Lysine equivalent in protein %	Vol. used for picrate cc.	Weight of picrate. Crop 1 g.	Weight of picrate. Crop 2 g.	Solubility correction, 0·54 g. picrate per 100 cc. mother-liquor g.	Explosion-point of picrate. Crop 1 °C.	Explosion-point of picrate. Crop 2 °C.	Nitrogen content of picrate. Crop 1. Theory 18·67 % %	Yield of lysine corrected %
1	250	11·84	6·18	246	21·252	2·445*	0·351	266	257	—	4·97
2	500	1·012	7·28	480	7·658	0·600†	0·110	264·5	256	18·50	4·69
3	500	0·846	7·3	480	7·194	0·283	0·089	265	250	18·49	4·78

* This weight includes 0·899 g. of pure lysine picrate obtained from the mother-liquor of the crude picrate after carrying the material through another phosphotungstic acid precipitation.
† This weight includes 0·137 g. of pure lysine picrate obtained as above.

by treatment with copper hydroxide [Vickery and Leavenworth, 1929], and the precipitates of histidine silver were decomposed with hydrogen sulphide rather than with hydrochloric acid. The full data are shown in Tables II–IV.

Third analysis. The material analysed had been crystallised four times from ammonium sulphate solutions and was then dialysed and subsequently coagulated by being poured into a large volume of alcohol. The coagulum was washed with absolute alcohol and ether and was dried in the air. It contained 15·61 % of nitrogen calculated moisture- and ash-free. The moisture content was 6·85 % and the ash was 0·22 %. Dr du Vigneaud's label declared the presence of a trace of sulphate. Distillation with barium hydroxide *in vacuo* showed that the contamination with ammonium sulphate was of the order 0·003 % and could therefore be neglected. The corrected weight of the quantity taken for analysis was determined from the weight of the dry material and by analysis of an aliquot part of the hydrolysate. The two results were 64·39 g. and 64·10 g., and the average value of 64·25 g. was employed in the calculations.

The protein was boiled for 30 hours with 900 cc. of 6*N* hydrochloric acid and the subsequent procedure was exactly the same as that of the second analysis. The data obtained are given in Tables II–IV.

Discussion.

The data collected in Table I show five closely agreeing determinations of the proportion of histidine yielded by crystalline egg-albumin. These were conducted by three different operators and three different preparations of the protein were employed; furthermore the procedures used differed in minor points. The conclusion seems justified therefore that the average of these five determinations, which is 1·42 %, represents very closely the correct yield of histidine from this protein. If the molecular weight of egg-albumin be taken at 34,000, and it be assumed that the protein contains 3 histidyl radicals per molecule, the proportion of histidyl should be 1·21 %. The yield of 1·42 % of histidine is equivalent to 1·25 % of histidyl and this agrees closely with the assumption. There seems little reason to doubt, therefore, that the molecule of egg-albumin contains three histidyl radicals.

The three arginine determinations carried out in this laboratory are all somewhat higher than those of Calvery [1932], the average being 5·39 %. They represent the results of two operators working independently and employing somewhat different procedures. Calculated as arginyl, the result is equivalent to 4·83 %. The theoretical requirements for 10 arginyl radicals per molecule of egg-albumin is 4·59 %, that for 11 radicals is 5·05 %. Our highest figure for arginine, 5·56 %, when calculated as arginyl, is equivalent to 4·98 % and the agreement between this figure and the requirement for 11 arginyl radicals is strikingly close. Our results are best interpreted, then, by the assumption that the molecule of egg-albumin contains 11 arginyl radicals.

The determination of lysine is probably the least trustworthy of that of any of the bases, and it is in this determination that the advantage of working on a large scale becomes manifest. We regard the result of our first analysis, 4·97 %, as probably nearest the truth, the somewhat lower figures of the other analyses serving to confirm the order of magnitude. Calculated as lysyl, this is equivalent to 4·36 %; the theoretical requirements of 11 and 12 lysyl radicals are 4·14 % and 4·52 % respectively. The most probable value for the lysyl content of egg-albumin, according to the present analysis, is therefore 12 radicals.

Summary.

The proportions of the basic amino-acids yielded by crystalline hen's egg-albumin, after acid hydrolysis, have been found to be histidine 1·42 %, arginine 5·39 %, and lysine 4·97 %. These results are in close agreement with the assumption that the molecule of egg-albumin, the weight of which is probably close to 34,000, contains 3 histidyl radicals, 11 arginyl radicals, and 12 lysyl radicals.

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